STUDIES ON THE MECHANISM OF ACTION OF ARGININE KINASE

A THESIS

submitted for the Degree

of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

ELIZABETH SMITH

December, 1969

This thesis embodies the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from October, 1966 to December, 1969, during the tenure of an Australian National University Research Scholarship.

STATEMENT

In accordance with the regulations of the Australian National University a statement describing which parts of the work in this thesis have been carried out by myself is required.

Apart from the results reported in Chapter V, which were obtained in collaboration with Dr. W.J. O'Sullivan, Department of Medicine, University of Sydney, and the polyacrylamide gel electrophoresis, ultracentrifuge analysis and amino acid analysis, which are acknowledged in the text, all experimental results were obtained by myself.

Elizabeth Smith

Elizabeth Smith

ACKNOWLEDGEMENTS

I am most grateful to my supervisor, Dr. J.F. Morrison, for his interest and encouragement during this work, and for his readiness for discussion at all times.

I should like to thank Professor F.W.E. Gibson for the privilege of working in the department, and to express my appreciation to members of the department for help they have given me. In particular, my thanks are due to Mrs. M. Labutis for her most willing and skilful technical assistance and to the Head Technicians, Mr. I.K. Reid and Mr. A.B. Howkins, for their co-operation in many matters.

My sincere thanks go to Dr. E. Heyde for her careful and constructive criticism of drafts of this thesis, to Dr. W.J. O'Sullivan for his collaboration in the work reported in Chapter V and to Dr. D.C. Shaw for his assistance with the work reported in Chapter VI.

Further, I should like to thank the staff of the photographic department for the preparation of thesis prints, and to express my gratitude to Mrs. C. Withers for her rapid and accurate typing of this thesis. In addition, I wish to thank my husband, Ben, for his assistance.

I am also grateful for the award of an Australian National University Scholarship, during the tenure of which this work was carried out.

PREFACE

Equations and tables in the Introduction are identified by Arabic numerals only, while those in the following Chapters (I to VI) consist of two parts : Roman numerals indicating the chapter, and Arabic numerals the number of the equation, figure or table within the chapter.

Abbreviations used in this thesis are :

AMP, ADP and ATP	adenosine mono-, di- and triphosphate
dADP and dATP	deoxyadenosine di- and triphosphate
$AMP-CH_2-P$	phosphonate analogue of ADP
IDP and ITP	inosine di- and triphosphate
UDP	uridine diphosphate
GDP	guanosine diphosphate
CDP	cytidine diphosphate
PP_i	inorganic pyrophosphate
PA	phosphoarginine
NAD and NADH	oxidised and reduced nicotinamide adenine dinucleotide
NADP and NADPH	oxidised and reduced nicotinamide adenine dinucleotide phosphate
EDTA	ethylene diamine tetraacetic acid

DCTA — trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid

DTNB — 5,5'-dithiobis-(2-dinitrobenzoic acid)

In addition, abbreviations have been used to describe the computer programmes of Cleland (1963d) which have been used in the analysis of kinetic data. The equations to which the programmes fit the data, as well as the abbreviated names of these programmes, are listed below.

No.	Title	Name	Equation
1.	HYPER	hyperbola	$v = \frac{VA}{K + A}$
2.	LINE	line	$y = ax + b$
3.	PARA	parabola	$y = a + bx + cx^2$
4.	PINGPONG	ping pong mechanism	$v = \frac{VAB}{K_a B + K_b A + AB}$
5.	SEQUEN	sequential mechanism	$v = \frac{VAB}{K_{ia} K_b + K_a B + K_b A + AB}$
6.	COMP	competitive inhibition	$v = \frac{VA}{K\left(1 + \frac{I}{K_i}\right) + A}$
7.	NONCOMP	non-competitive inhibition	$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)}$

No.	Title	Name	Equation
8.	PARACOMP	slope parabolic competitive inhibition	$v = \frac{VA}{K\left(1 + \frac{I}{K_{I1}} + \frac{I^2}{K_{I1}K_{I2}}\right) + A}$
9.	SUBINH	substrate inhibition	$v = \frac{VA}{K + A\left(1 + \frac{A}{K_i}\right)}$

The NUCALC computer programme was used to calculate the concentrations of the various ionic species from the total concentrations of reactants and metal ion, and their stability constants (Morrison and Cleland, 1966).

SYNOPSIS

Arginine kinase (E.C.2.7.3.3), which catalyses the reversible reaction of ATP and arginine to form ADP and phosphoarginine, was isolated from the tail muscle of the West Australian sea-water crayfish, Panulirus longipes. A high degree of purity was demonstrated by starch gel electrophoresis, polyacrylamide gel electrophoresis and Sephadex chromatography.

Kinetic investigations of the reaction mechanism of the enzyme involved initial velocity, product inhibition, isotope exchange and dead-end inhibition studies, as well as the use of substrate analogues, both as substrates and as dead-end inhibitors. All kinetic studies were carried out assuming the metal-nucleotide complexes and the free guanidino compounds to be the true substrates of the reaction, and the concentration of the free metal ion, usually magnesium, was held constant. The results indicate that the reaction mechanism is rapid equilibrium, random, with two dead-end complexes, viz., enzyme-MgADP-arginine and enzyme-MgATP-phosphoarginine. Further support for this mechanism was obtained from nuclear magnetic resonance studies on arginine kinase, with manganese as the activating ion.

Sedimentation velocity ultracentrifuge analysis suggested a molecular weight of approximately 40,000. Amino acid analysis and peptide mapping of the enzyme were also carried out and the results indicate that the enzyme is not composed of subunits. The importance of sulphydryl groups for the catalytic activity of this arginine kinase was demonstrated by inactivation experiments with iodoacetamide, using substrates to protect the enzyme from inactivation.

The results of these investigations indicate that the mechanism of action of the arginine kinase from P. longipes is similar to that of creatine kinase, the phosphagen phosphotransferase present in vertebrate muscle.

TABLE OF CONTENTS

INTRODUCTION

The work reported in this thesis is concerned with the elucidation of the mechanism of the reaction catalysed by arginine kinase from P. longipes, and a study of some of the properties of the enzyme. A number of methods of approach to the problem of the reaction mechanism have been used, and the Introduction comprises some background to the techniques employed. Since the major part of the investigation has involved kinetic methods, the uses and limitations of these in the study of bireactant mechanisms are discussed. Also, the application of some physical and chemical methods of elucidating the mechanism of action of an enzyme is outlined, and reference made to some phosphotransferases whose reaction mechanisms have been determined. The rationale for research into the properties of this arginine kinase is also given, with some discussion of previous studies of arginine kinases from a number of sources.

* * * * * * * * * * *

Since virtually all the chemical reactions occurring in living organisms are catalysed by enzymes, it is clear that these processes cannot be fully

understood until the mechanisms of enzymic catalysis have been determined. Therefore, a great deal of biochemical research has been directed towards elucidating the mechanisms of enzyme action, with the object of explaining why these proteins are capable of accelerating specific chemical reactions. An important step towards the understanding of the mechanism of action of an enzyme is the determination of the order of addition of substrates and release of products. In this thesis the term "reaction mechanism" will be used to denote this level of understanding of the processes catalysed by the enzyme. One valuable method available for the study of the reaction mechanism is enzyme kinetics, which has been used for many years, though its potential value has been exploited only during the last decade.

The development of enzyme kinetics

The development of the subject of enzyme kinetics has been fully discussed by Segal (1959). The earliest work directly involving attempts to use enzyme kinetics in the investigation of enzyme action was that of Brown (1902). His ideas were elaborated by Henri (1903), who reported a method for deriving the initial rate equation for an enzymic reaction. This equation was

further developed by Michaelis and Menten (1913) who, like Henri, assumed the enzyme and enzyme-substrate complex to be in rapid equilibrium. Their initial rate equation with one substrate was :-

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (1)$$

where v is the initial velocity, [S] is the substrate concentration, V_{max} is the maximum velocity with saturating substrate, and K_m, the Michaelis constant, is the substrate concentration required to achieve half maximum velocity. Because of the rapid equilibrium assumption, K_m also represents the dissociation constant for the enzyme-substrate complex. Bodenstein (1913) introduced the concept of steady state, which permits an expression for the concentration of intermediates without the rapid equilibrium assumption. Briggs and Haldane (1925) realised that the steady state condition is reached very quickly after the start of an enzymic reaction, and further developed this concept. Although, under steady state conditions, the Michaelis constant, K_m, does not represent a dissociation constant, the Michaelis-Menten equation, (1), remains the initial rate equation for a single-substrate reaction.

A further important step in the development of enzyme kinetics was the introduction by Lineweaver and Burk (1934) of the double reciprocal plot of initial velocity against substrate concentration. This allows clear qualitative presentation of kinetic data, although, as pointed out by Dowd and Riggs (1965), this type of plot provides a less accurate method of graphically determining the values of K_m and V_{max} than the other two forms of linear transformation, which involve plotting S/v against S, or v against v/S. However, because the kinetic constants can be determined accurately by mathematical analysis, the Lineweaver-Burk plot remains a most satisfactory way of presenting kinetic data, since it is relatively easy to arrange the more complex rate equations in double reciprocal form. The method was extended to two-substrate reactions by Florini and Vestling (1957).

It must be emphasised that the early work on enzyme kinetics was concerned only with single-substrate reactions, and no consideration was given to the subject of product release. For such reactions, the order of release of products can easily be determined by product inhibition studies. However, this discussion will be restricted to the kinetics of bireactant mechanisms,

involving two substrates and two products, for which there are a larger number of possible mechanisms because the order of both substrate addition and product release may vary. For many years two-substrate reactions were regarded as necessarily proceeding via the ordered formation of a ternary complex of the two substrates with the enzyme (Woolf, 1931). Later, however, it was realised that enzyme reactions involving two substrates could occur in a number of ways. Alberty (1953) derived initial velocity equations to describe a number of mechanisms, and showed that they could not be distinguished by initial velocity studies alone. An important contribution to the field of enzyme kinetics was the introduction, by King and Altmann (1956), of a technique which greatly simplifies the derivation of rate equations for enzymic mechanisms. The method has been used extensively by Cleland (1963a, 1963b) to derive complete rate equations.

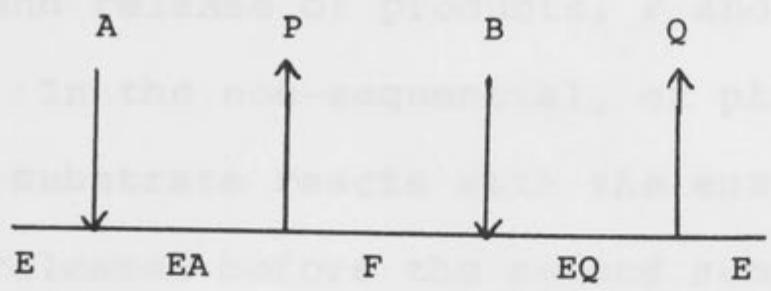
The kinetic study of bireactant enzymes

A bireactant enzyme may have a non-sequential reaction mechanism or one of three types of sequential reaction mechanism. These are illustrated on the following page, in the form introduced by Cleland (1963a), where the line represents the enzyme, E,

Examples

NON-SEQUENTIAL

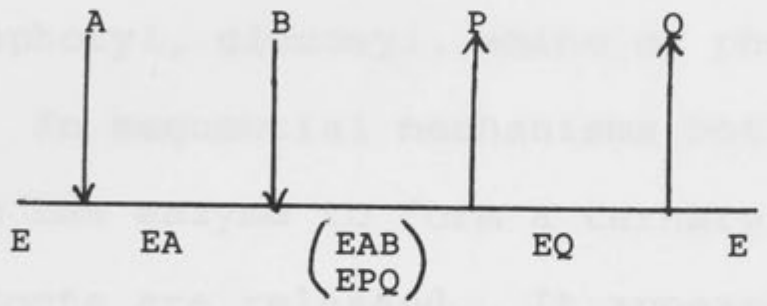
Ping pong



Glutamic oxaloacetate transaminase (Henson & Cleland, 1964)

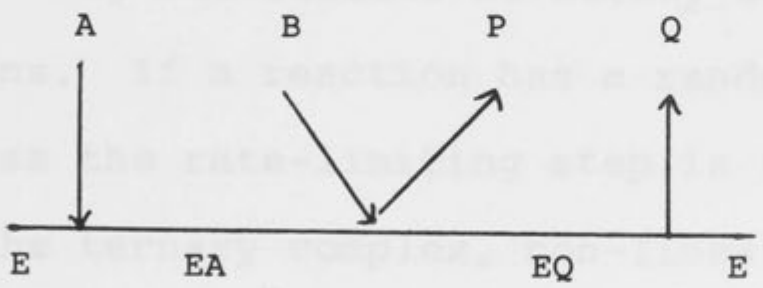
SEQUENTIAL

Ordered



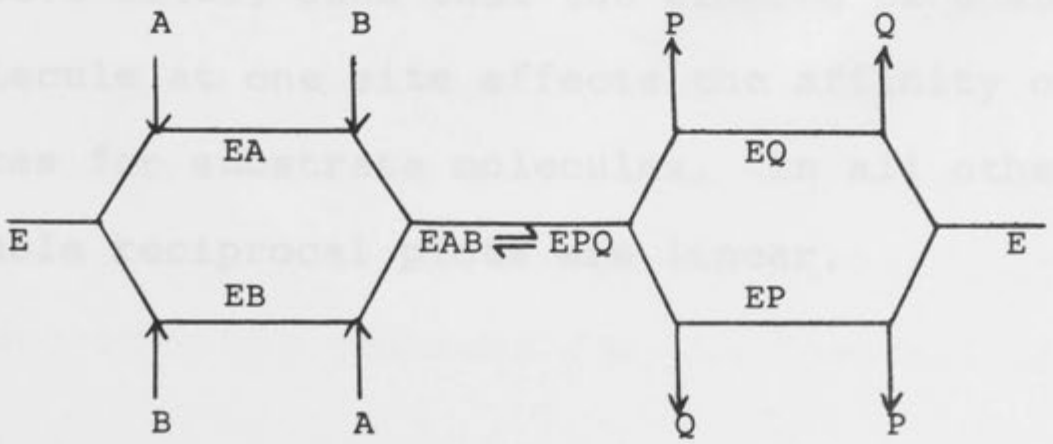
Alcohol dehydrogenase (Wratten & Cleland, 1963)

Theorell-Chance



Lactate dehydrogenase (Iso Theorell-Chance) (Zewe & Fromm, 1965)

Random (Rapid Equilibrium)



Creatine kinase (See Table 1 for references)

and the arrows show the addition of substrates, A and B, and release of products, P and Q.

In the non-sequential, or ping pong, mechanism one substrate reacts with the enzyme and one product is released before the second substrate adds to the enzyme. Therefore, the enzyme exists in two stable forms; as the free enzyme, E, and as some complex, F, of the enzyme with a transferable moiety such as a phosphoryl, glucosyl, amino or phosphoribosyl group.

In sequential mechanisms both substrates react with the enzyme to form a ternary complex before any products are released. It appears that most, if not all, random reactions are in rapid equilibrium, as judged by the results of steady state kinetic investigations. If a reaction has a random mechanism, then, unless the rate-limiting step is the interconversion of the ternary complex, non-linear double reciprocal plots are obtained (Alberty, 1953). Non-linear plots are also obtained when an enzyme has a number of active sites, such that the binding of a substrate molecule at one site affects the affinity of the other sites for substrate molecules. In all other cases double reciprocal plots are linear.

Initial velocity studies alone can usually be used to distinguish between ping pong and sequential mechanisms. Double reciprocal plots of initial velocity against the concentration of one substrate, with the other substrate held at a series of fixed concentrations, yield families of parallel lines when the mechanism is ping pong. When the reaction mechanism is sequential, whether Ordered, rapid equilibrium random or Theorell-Chance, a series of converging lines is obtained. However, under circumstances where the assay is relatively insensitive, so that the two substrates can be varied only over a range which is high in relation to one or more of the kinetic constants, parallel lines may be obtained, even though the reaction mechanism is sequential (Toews, 1966; Henderson et al., 1968).

The reaction mechanism can usually be qualitatively determined by product inhibition studies (Cleland, 1963c). The product of a reaction may combine with the enzyme form with which it normally reacts as a substrate. Also, it may react with another enzyme form to give a dead-end complex, which is a complex that cannot break down to give products. The various mechanisms give different product inhibition patterns, except that the patterns for the Theorell-Chance

mechanism and the rapid equilibrium random mechanism, with two dead-end complexes, are qualitatively similar. Quantitatively, these two mechanisms can be distinguished by comparing the values of the apparent kinetic constants from the competitive inhibition patterns, as a function of the concentration of the fixed substrate. If the mechanism is rapid equilibrium random, the value of the apparent kinetic constant varies significantly with changes in the fixed substrate concentration. For a Theorell-Chance mechanism, however, changes in the fixed substrate concentration do not affect the value of the constant. Thus, enzyme kinetics can yield information about the order of addition and release of reactants, whether random or obligatory, and, consequently, the nature of enzyme-reactant complexes can be deduced.

Steady state kinetics cannot, however, reveal anything about the isomerisation of the central complex in a sequential reaction mechanism. Any number of ternary complexes may exist, but it is usually assumed that only one is present, since the form of the initial velocity equation does not depend on this factor, although the meaning of certain kinetic constants does. From quantitative product inhibition studies it is

sometimes possible to detect isomerisation of stable enzyme forms, which are those forms that can only isomerise or add reactants (Cleland, 1963a).

Another important tool in the kinetic investigation of bireactant enzyme mechanisms is the use of dead-end inhibitors. The dead-end inhibition patterns obtained with substrate analogues, which are not substrates for the reaction but act as inhibitors, can also give information about the order of addition of substrates (Cleland, 1963b).

The results of any kinetic investigation must be interpreted with caution, since they may be consistent with more than one mechanism. It is essential, therefore, to consider as many mechanisms as possible and determine those which fit all the available data. Thus, the kinetic study of a reaction can show that some mechanisms are not feasible, but cannot determine conclusively that the reaction proceeds by a particular mechanism. For this reason, it is advisable to use additional techniques to confirm and define the enzymic reaction mechanism. One such technique, isotope exchange, is now widely recognised as an adjunct to kinetic studies in the elucidation of enzyme mechanisms, and is discussed below.

Isotope exchange studies

The technique of measuring rates of isotope exchange and incorporation in enzyme-catalysed reactions was first used by Doudoroff et al. (1947) and was developed by Boyer (1959). Since that time, isotope exchange techniques have been widely used in the determination of reaction mechanisms, as illustrated in the table showing some of the phosphotransferases whose mechanisms have been investigated (Table 1).

There are two major uses of isotope exchange in elucidating the reaction mechanisms of bireactant enzymes. Firstly, a ping pong mechanism can be demonstrated by allowing the enzyme to react with a radioactive substrate, in the absence of the other substrate. If the label can then be detected in the product, a partial reaction must have occurred. However, it is important to measure the initial rate of isotope incorporation into the product under these conditions, and compare it with that calculated using kinetic constants obtained from initial velocity and product inhibition studies.

The other valuable technique, using labelled reactants, is isotope exchange at equilibrium in sequential reactions. The method of investigation is to allow the reaction to reach equilibrium and, then,

to add a small amount of a radioactive reactant, insignificant in relation to the equilibrium concentration of the non-radioactive compound. The slight perturbation of the equilibrium, which results, is not sufficient to change the rate of equilibrium exchange between the pair of reactants. Therefore, by measuring the initial rate at which the second reactant of the pair is labelled, it is possible to determine the rate of isotope exchange at equilibrium. This type of experiment may be extended by varying the concentrations of one pair of reactants in constant ratio, thus maintaining the equilibrium condition, and measuring the initial rates of exchange at different equilibrium concentrations of the reactants. The pattern obtained depends on the reaction mechanism of the enzyme, so this method can be used to identify a particular reaction mechanism (Morrison and Cleland, 1966; Su and Russell, 1968; Henderson et al., 1968). Another very important use of isotope exchange studies at equilibrium is in confirming that a reaction mechanism is truly rapid equilibrium, since it is difficult to detect the slight non-linearity of double reciprocal plots, obtained in kinetic studies, when the reaction mechanism only approximates to rapid equilibrium random.

For a truly rapid equilibrium random mechanism, the initial rates of isotope exchange at equilibrium for both pairs of reactants should be identical. This has been demonstrated for creatine kinase by Morrison and Cleland (1966). For galactokinase, however, kinetic and isotope exchange studies yielded results which were not in complete agreement (Gulbinsky and Cleland, 1968). Initial velocity and product inhibition studies indicated that the reaction mechanism of this enzyme was rapid equilibrium random, with two dead-end complexes. Isotope exchange studies at equilibrium, on the other hand, showed that the rate of MgATP-MgADP exchange was 2.5 times faster than the galactose—galactose-1-phosphate exchange, under a variety of conditions, indicating that the mechanism is not truly rapid equilibrium. The use of isotope exchange techniques can, therefore, provide more definitive evidence as to whether a mechanism is truly rapid equilibrium than can be obtained from kinetic studies alone.

Use of physical and chemical methods in elucidating the reaction mechanisms of enzymes

As has been discussed by Lumry (1959), there are very large gaps in our knowledge of the actual chemical events occurring at the active site of an enzyme. Even

for those few enzymes whose complete three-dimensional structure has been determined at the atomic level, it is difficult to understand the molecular processes of catalysis (Perutz, 1969). However, there are physical and chemical approaches, which can provide useful information about the active centre of the enzyme and confirm conclusions drawn from kinetic investigations.

Direct binding studies, by equilibrium dialysis, can be used to determine whether the addition of substrates to an enzyme is ordered or random, and to measure the binding constants for the combination of substrates with the enzyme. The values obtained using these techniques can be compared with those obtained from kinetic studies.

Another approach, if the reaction mechanism is thought to be ping pong, is to isolate the stable complex of enzyme and a radioactive transferable group, which is formed after the release of the first product. This can be done by phenol extraction of a phosphorylated enzyme (Boyer and Bieber, 1967), or by less drastic methods, such as column chromatography. If such a complex can be isolated without inactivation of the enzyme, then it can be allowed to react with the second substrate of the reaction, to form the

second product (Norman et al., 1965; Dowler and Nakada, 1968; Grinnell and Nishimura, 1969). This provides direct evidence for a ping pong mechanism, but gives no information about the relative dissociation constants of the reactants. However, dissociation constants can be determined from kinetic studies of the partial exchange reactions (Cleland, 1967).

Nuclear magnetic resonance (NMR) techniques, which involve the measurement of proton relaxation rates of water (PRR), and electron paramagnetic resonance (EPR), have been developed by Cohn (1963, 1967) for the study of protein function. These approaches to the elucidation of enzyme reaction mechanisms require that the enzyme is metal-activated, and that a paramagnetic ion, such as Mn^{2+}, can be used as the activating ion. Since this is true for phosphotransferases, the method has been used in the study of several enzymes belonging to this group. It has been possible to show whether the metal ion binds directly to the enzyme, as is the case with pyruvate kinase (Cohn, 1963; Mildvan and Cohn, 1965), or is involved as the metal complex of the nucleotide substrate of the reaction, as with creatine kinase (O'Sullivan and Cohn, 1966a). NMR techniques can also be used in some

circumstances to determine whether the addition of substrates is random or ordered and, indirectly, whether ternary complexes may be formed. Quantitative results of NMR studies yield dissociation constants for enzyme-substrate interactions, and the values of these may be compared with the results of kinetic and binding experiments.

Yet another method used in the elucidation of the mechanisms of enzyme action is inactivation of the enzyme, either by heat treatment or by use of a chemical which reacts specifically with a particular amino acid residue, such as cysteine, histidine or lysine. It is then possible to determine which substrates or substrate analogues, either singly or in combinations giving dead-end complexes, protect the enzyme from inactivation by a specific reagent. Thus, one can deduce which amino acid residues are essential for the catalytic process, either because they are present at the active site or because of their ability to maintain the ternary structure of the enzyme, so that the catalytic centre is in the correct conformation for enzymic activity. Also, by the use of radioactive reagents, which inactivate the enzyme by combination with a specific amino acid, it is possible to determine

the amino acid sequence around this residue. This is achieved by allowing the enzyme to react with the labelled reagent, breaking the protein down into peptides by tryptic digestion, and separating the peptides by electrophoresis or chromatography, or both, so that a peptide map is obtained. By autoradiography one can detect the peptide(s) which have been labelled with the radioactive compound. The amino acids near a labelled residue can then be identified by determining the amino acid composition and sequence of the radioactive peptide. However, unless a complete atomic model of the protein has been constructed from X-ray crystallography data and the results of sequence determinations, this gives little information about the actual chemical structure of the catalytic centre. This is because other catalytically active amino acids are not necessarily close to the labelled residue in the primary structure, though by folding of the amino acid chain they may be brought together. The information is, however, useful in comparative studies of the active sites of enzymes.

An important point, which has been emphasised by Perutz (1969), is that in all enzymes whose complete structure has been determined by X-ray crystallography,

and these are mostly hydrolytic enzymes, the interior of the molecule is non-polar, and this hydrophobic area extends to the surface at the catalytic centre. Therefore, the medium in which catalysis occurs has a low dielectric constant, so that electrical interactions involved in substrate binding and catalysis are much stronger at the active centre, which is in a cleft or indentation of the molecule, than they would be in an aqueous medium.

Thus, the goal in an investigation of the mechanism of action of an enzyme must be, in part, to determine by kinetic and thermodynamic means the order of binding and release of reactants, and the affinity of the enzyme for these reactants. In addition, however, knowledge of the chemical environment at the active site, and of the chemical events occurring during catalysis, is essential for complete understanding of the mechanism of an enzymic catalysis.

Mechanisms of phosphotransferase reactions

Phosphotransferases are enzymes which catalyse the transfer of a γ-phosphoryl group of nucleoside triphosphates to some acceptor molecule, with the formation of the corresponding nucleoside diphosphate (Bock, 1960). The most common nucleoside triphosphate

substrate for such enzymes is adenosine triphosphate (ATP). From studies with O^{18}-ATP it has been deduced that cleavage occurs between the terminal P and the bridge oxygen (Cohn, 1959; Bock, 1960).

Biologically, the phosphotransferases are a very important group of enzymes, since they catalyse the reaction of ATP, the main source of high energy phosphate in the cell, with acceptor molecules for the phosphoryl group. These reactions result in the formation of phosphoryl compounds, such as glucose-6-phosphate, phosphoenol pyruvate and galactose-1-phosphate, which are key molecules in intermediary metabolism, as well as the high energy storage compounds phosphocreatine and phosphoarginine, in vertebrate and invertebrate muscle respectively. In the lower organisms there are a number of other guanidines, which act as phosphate acceptors to form high energy storage compounds, known as phosphagens. These include glycocyamine, tauro-cyamine, lombricine, hypotaurocyamine and opheline (Thoai et al., 1953; Thoai and Robin, 1954; Robin and Thoai, 1962; Thoai et al., 1963), and specific enzymes exist for the formation and breakdown of the phosphoryl-ated forms of these guanidines.

To date several phosphotransferases have been investigated by kinetic and other techniques, and reaction mechanisms have been proposed. A summary of the proposed mechanisms for some of these phosphotransferases is given in Table 1, with indications of the methods used in their elucidation. From this table it is clear that there is no general pattern of reaction mechanisms of phosphotransferases. Thus, although reactions catalysed may involve similar chemical changes, there is not necessarily a similarity in the mechanism of catalysis, since this is the property of the specific enzyme catalysing the reaction. It has been found, however, that all the phosphotransferases investigated have a requirement for a divalent metal ion. This metal ion requirement has been discussed previously with regard to the function of the metal ion in forming a metal-nucleotide complex, which is a substrate of the reaction, or an active metal-enzyme complex. In a kinetic study of a phosphotransferase the concentration of the metal-nucleotide complex, when this is the true substrate of the enzyme, must be calculated. Also, the concentration of free metal ion must be held at some fixed value which is not inhibitory. The apparent stability constants for complexes of ATP and ADP with

Table 1. Summary of proposed mechanisms for some phosphotransferases

Enzyme	Mechanism	Methods	References
Creatine kinase (E.C.2.7.3.2)	Rapid equilibrium, random, with two dead-end complexes	Initial velocity studies Product inhibition studies	Morrison and James (1965)
		Dead-end inhibition studies Alternative substrate studies	James and Morrison (1966a, 1966b)
		Isotope exchange studies	Morrison and Cleland (1966)
		NMR studies	O'Sullivan and Cohn (1966a)
		Binding studies	Mahowald et al. (1962)
Hexokinase (E.C.2.7.1.1) (a) Yeast	Rapid equilibrium, random	Initial velocity studies Product inhibition studies Isotope exchange studies at equilibrium	Fromm and Zewe (1962) Zewe et al. (1964) Fromm et al. (1964) Ottolenghi (1964) Fromm (1969)
	Ordered	Phosphoryl group acceptor properties in presence of glucose	Hass et al. (1961)
		Initial velocity studies Product inhibition studies	Hammes and Kochavi (1962a, 1962b)
		NMR studies	Cohn (1963)
		Product inhibition studies	Noat et al. (1968)
(b) Skeletal muscle	Ordered	Initial velocity studies Product inhibition studies	Toews, C.J. (1966)
Galactokinase (E.C.2.7.1.6)	Rapid equilibrium, random, with two dead-end complexes	Initial velocity studies Product inhibition studies Isotope exchange at equilibrium	Gulbinsky and Cleland (1968)
Glucokinase (E.C.2.7.1.2)	Rapid equilibrium, random	Initial velocity studies Product inhibition studies	Parry and Walker (1966, 1967)

Table 1. (cont'd.)

Enzyme	Mechanism	Methods	References
Pyruvate kinase (E.C.2.7.1.40)	Sequential, probably rapid equilibrium, random	Initial velocity studies Product inhibition studies	Reynard et al. (1961)
Phosphoramidate-ADP phosphotransferase	Ping pong	Isolation of phosphoenzyme intermediate	Dowler and Nakada (1968)
Myokinase (E.C.2.7.4.3)			
(a) Rabbit muscle	Random with one dead-end complex	Initial velocity studies Isotope exchange studies Dead-end inhibition studies	Noda (1958) Rhoads and Lowenstein (1968)
(b) Yeast	Rapid equilibrium, random	Isotope exchange studies at equilibrium	Su and Russell (1968)
Nucleoside diphosphokinase (E.C.2.7.4.6)			
(a) Erythrocytic	Ping pong	Initial velocity studies Dead-end inhibition studies	Mourad and Parks (1965, 1966)
(b) Mitochondrial	Ping pong	Initial velocity studies Isolation of phosphorylated enzyme	Colomb et al. (1966) Goffeau et al. (1967) Norman et al. (1965)
(c) Yeast	Ping pong	Initial velocity studies Product inhibition studies Isotope exchange studies Isolation of phosphorylated enzyme	Garces and Cleland (1969)
Arginine kinase (E.C.2.7.3.3)			
(a) H. vulgaris	Sequential	Initial velocity studies NMR studies	Virden et al. (1965) O'Sullivan et al. (1969)

Table 1. (cont'd.)

Enzyme	Mechanism	Methods	References
Arginine kinase (E.C.2.7.3.3) (b) *J. verreauxi*	Ping pong	Initial velocity studies Partial isotope exchange reactions	Uhr *et al.* (1966)
Hypoxanthine-guanine phosphoribosyl phosphotransferase (E.C.2.4.2.8)	Ordered	Initial velocity studies Product inhibition studies Isotope exchange studies	Henderson *et al.* (1968)

Mg^{2+}, Mn^{2+}, and Ca^{2+}, and for complexes of some other compounds with Mg^{2+}, have been determined by O'Sullivan and Perrin (1964) under conditions suitable for kinetic studies. Therefore, it is possible to vary the concentration of these complexes, while maintaining the concentration of the free metal ion at a fixed value. This results, however, in variable concentrations of the free nucleotide, which may be an inhibitory species, so it is advisable to maintain the concentration of the free nucleotide as low as possible.

Distribution of phosphagens and their phosphotransferases

There has been considerable interest in the possible phylogenetic significance of phosphagens since the suggestion by Kutscher and Ackerman (1926) that the animal kingdom could be divided into two distinct classes on a chemical basis, according to whether they contain creatine or not. These classes, the Creatinata containing creatine, and the Acreatinata without creatine, would be roughly equivalent to the vertebrates and the invertebrates, respectively. However, since that time, a considerable amount of research on the distribution of phosphagens in various species has been reported. It has been shown that the division suggested by Kutscher and Ackerman would be an over-simplification

of the position, which is now known to be very complex, with some invertebrates containing both creatine and arginine (Moreland et al., 1967). Also, phosphoarginine has been detected in some primitive chordates, the Hemichordata (Needham et al., 1932; Baldwin and Yudkin, 1950), phosphocreatine is present in a number of invertebrates (Baldwin and Yudkin, 1950; Yudkin, 1954; Roche and Robin, 1954), and there are at least five other phosphagens whose existence in the phylum Annelida has been definitely established (Thoai et al., 1953; Robin and Roche, 1954; Thoai and Robin, 1954). Because of the complexity of the situation revealed at that stage in the development of the subject, Ennor and Morrison (1958) concluded that studies of the distribution of phosphagens and their phosphotransferases could not contribute a great deal to the understanding of evolutionary processes. During the past decade, however, there has been renewed interest in the phosphagens and phosphagen phosphotransferases and, although no clear pattern has yet emerged, there are indications that detailed comparative studies of these systems in various organisms may provide information about the evolution of members of the species concerned.

Moreland et al. (1967) have carried out a detailed survey of phosphagens and their phosphotransferases with regard to the evolutionary relationships within the phylum Echinodermata, in which creatine, arginine and glycocyamine phosphotransferases are present. They conclude that creatine kinase has emerged from the gamete of a common ancestral species by parallel evolution, to occur in the forms now present in echinoderms. These authors have also put forward an interesting hypothesis, after investigation of the distribution of phosphagens and their kinases among the Echinoidea, a class within the Echinodermata. In this study they determined that arginine kinase was present in the muscle of all but one of the twenty one species investigated, and that the same enzyme occurred in the eggs of these organisms. In the sperm, however, creatine kinase was present in all of the species investigated. The authors suggest that an organism could acquire a selective advantage if cells, such as the motile gamete, which contain considerable stores of phosphagen, utilise for the synthesis of this phosphagen a compound not required in their other metabolic processes. This is particularly true in the testes where the synthesis of nuclear histones, con-

taining large amounts of basic amino acids, would deplete the reserves of arginine, thus displacing the substrate equilibrium in favour of arginine, and causing breakdown of phosphoarginine. The use of phosphocreatine as the phosphagen, however, serves to isolate the energy storage system from the rest of the amino acid metabolism in the cell. By this means the equilibrium can be more readily maintained, even though arginine is a precursor of creatine, so that there is no real conservation of arginine by the use of phosphocreatine as the phosphagen.

Watts (1968) has reviewed the mass of evidence which has accumulated in relation to the evolutionary significance of the phosphagen phosphotransferases, and suggests that it supports the long-held view that arginine kinase, as the only phosphagen phosphotransferase to occur in the monomeric form, may be the most primitive of these enzymes. Further support for this proposal comes from the fact that no monomeric phosphotransferases have been detected in the chordate line, which is evolutionarily the most advanced. Thus, it is now considered that a dimeric phosphagen phosphotransferase may confer a selective advantage on the organism in which it occurs, and it appears that the

evolution of an arginine kinase dimer has occurred more than once among the Mollusca.

The overall significance of the distribution of phosphagens and their phosphotransferases with respect to evolution remains uncertain, and a great deal more work on the isolation and classification of phosphotransferases from many organisms will be required before it is possible to obtain any clear indication of their evolutionary significance. This aspect of the study of phosphagen phosphotransferases is of great interest, and it is unfortunate that, because so many organisms must be examined, it is impossible to make a detailed investigation of the properties of all the enzymes detected.

Function of the phosphagen phosphotransferases

The fact that phosphocreatine and phosphoarginine are the main energy storage compounds in vertebrates and invertebrates, respectively, has already been discussed. The chief biological function of creatine and arginine phosphotransferases appears to be in the formation and breakdown of the phosphagens (Morrison and Ennor, 1960). The problem of the role of phosphagen phosphotransferases has been discussed by Watts (1968), in an attempt to clarify the nature of

the selective forces which apparently tend to favour the evolution of a dimeric enzyme. It has been suggested that the reason for the evolution of creatine kinase in vertebrates may be that this enzyme participates in the actual contractile mechanism of the muscle (Carlson and Siger, 1960). Evidence supporting this hypothesis was presented by Yagi and Noda (1960), who showed that purified creatine kinase could phosphorylate myosin-bound ADP in the presence of phosphocreatine. Perry (1954) had previously shown that phosphocreatine and ADP, in the presence of creatine kinase, were more efficient than ATP as an energy source for the contraction of myofibrils. This indicated that, although ATP was the immediate source of energy for muscular contraction, the molecule was more effective when formed from ADP and phosphocreatine by creatine kinase. Yagi and Mase (1962) provided an explanation for this observation by demonstrating that creatine kinase binds with a very high dissociation constant (6×10^{-8} M) in a position very close to the myosin ATPase site, so that the ATP formed is immediately available as a substrate for the myosin ATPase.

Phosphagen phosphotransferases are present in many tissues other than muscle (Morrison and Ennor,

1960; Kuby and Noltmann, 1962) and their function is not confined to participation in muscular contraction. It seems likely that phosphagens are present in most types of tissue as energy storage compounds, and Wood (1963a,b) has purified creatine kinase from ox brain and compared some of its properties with those of the muscle enzyme. Also, Bessman and Fonyo (1966) found that creatine kinase bound to mitochondria in pigeon breast muscle requires extra-mitochondrial nucleotides, and suggested that this enzyme could be involved in the control of respiration in response to muscular activity.

Little is known about the physiological role of arginine kinase and its function in muscular contraction in invertebrates. Hanson and Lowy (1960), however, observe that there are marked variations in the muscle structure of invertebrates, and this may account, in part, for the differences among arginine kinases, which exist in monomeric, dimeric and tetrameric forms (Lacombe et al., 1969). Nevertheless, a number of studies on purified arginine kinases - chemical, kinetic and comparative - have been carried out with the enzyme purified from various sources, and some of these are discussed in the next section.

Some properties of arginine kinases

Since this thesis is concerned with a study of the mechanism of action of arginine kinase from the West Australian crayfish, Panulirus longipes, a brief discussion of previous work on arginine kinases from various sources will be given. Crystalline arginine kinase was first prepared by Szorenyi et al. (1949) from the muscle of a fresh-water crab, and later by Elodi and Szorenyi (1956) from the muscle of a different species of crab. Both these preparations involved only ammonium sulphate fractionation of the muscle extracts. The enzyme has since been isolated from the European lobster, Homarus vulgaris (Pradel et al., 1964; Pradel et al., 1965; Virden et al., 1965), the Australian sea-water crayfish, Jasus verreauxi (Morrison et al., 1957; Uhr et al., 1966), the American lobster, Homarus americanus (Blethen and Kaplan, 1967), the hermit crab, Pagurus bernhardus, the blue crab, Callectinus sapidus, and the horseshoe crab, Limulus polyphemus (Blethen and Kaplan, 1968), and the siponcle, Sipunculus nudus (Lacombe et al., 1969).

From their comparative studies on arthropod arginine kinases, Blethen and Kaplan (1968) have found

that the amino acid compositions of those which they have purified are very similar, even though the species in which they occur are widely divergent. Most of the comparative studies with arginine kinases have, however, been less detailed, enabling the authors to investigate the enzymes from a larger number of organisms (e.g. Moreland et al., 1967). Thus, there are two main approaches to the study of arginine kinase : firstly, the enzyme may be purified and its properties investigated in some detail; and, secondly, the enzymes from a large number of organisms may be examined less exhaustively in crude tissue extracts.

The most detailed studies of arginine kinases have been carried out on the enzymes from the European lobster, H. vulgaris (Virden et al., 1966; Virden and Watts, 1966a, 1966b; Kassab et al., 1968; Pradel and Kassab, 1968; Lacombe et al., 1969), the American lobster, H. americanus (Blethen and Kaplan, 1967), the Australian sea-water crayfish, J. verreauxi (Uhr et al., 1966) and the siponcle, S. nudus (Lacombe et al., 1969). The molecular weights of a number of arginine kinases have been determined and range from 36,000 to 43,000 for the enzymes obtained from crustacean sources. Arginine kinases from other organisms exist as monomers

of molecular weight approximately 40,000, dimers of molecular weight approximately 80,000 and tetramers of molecular weight approximately 160,000 (Robin et al., 1969). Lacombe et al. (1969) have designated these, Types I, II and III, respectively.

Inactivation studies with arginine kinase from H. americanus have shown that the enzyme is reversibly inactivated by 8 M urea (Blethen and Kaplan, 1967). Some amino acid residues essential for the activity of arginine kinase from H. vulgaris have been determined by inactivation studies using specific chemicals. Virden and Watts (1966b) have shown that thiol groups are essential for enzymic activity, by inactivation studies with iodoacetamide. Also an essential lysine residue has been demonstrated by Kassab et al. (1968), and Pradel and Kassab (1968) have presented evidence for a critical histidine, by reaction of the enzyme with a specific reagent, diethylpyrocarbonate. Thus, cysteine, lysine and histidine have all been implicated as essential for the activity of arginine kinase from H. vulgaris.

Some kinetic studies of arginine kinase have been carried out with the enzymes from H. vulgaris (Virden et al., 1965; Lacombe et al., 1969), J. verreauxi

(Uhr et al., 1966) and S. nudus (Lacombe et al., 1969). Whereas the results of initial velocity and product inhibition experiments, and the qualitative demonstration of partial isotope exchange reactions catalysed by the J. verreauxi enzyme were consistent with this enzyme having a ping pong mechanism (Uhr, 1966), the initial velocity studies with the enzyme from H. vulgaris indicated a sequential mechanism. Because the arginine kinases were purified from two different species of crustacean, there is no a priori reason for the reaction mechanisms of the enzymes to be similar. However, the arginine kinases from H. vulgaris and J. verreauxi appear to be similar in their molecular weights, and both are sensitive to sulphydryl reagents (Virden et al., 1966; Morrison et al., 1957; Virden and Watts, 1966b). Since, at the time at which this work was commenced, neither kinetic study was exhaustive, it seemed that it might be profitable to carry out a more detailed investigation of an arginine kinase from a different source, employing a wider range of experimental methods. The enzyme chosen for this was the arginine kinase from the tail muscle of the West Australian sea-water crayfish, P. longipes.

CHAPTER I

The Purification of Arginine Kinase and Some Criteria of Purity

INTRODUCTION

Since the West Australian crayfish, P. longipes, is closely related to J. verreauxi (Dakin, 1952, p.183), it seemed probable that the arginine kinases from the two species could be purified by similar methods. Uhr et al. (1966) have reported a purification procedure for arginine kinase from J. verreauxi, and the methods used in the purification of the enzymes from the European lobster (Virden et al., 1965) and the siponcle (Lacombe et al., 1969) are based on similar procedures. Virden et al. (1965) found that increasing concentrations of salts, such as sodium chloride, sodium acetate, and sodium or potassium nitrate inhibit the H. vulgaris arginine kinase. Also, Uhr (1966) showed that several anions, including chlorate and nitrate, have inhibitory effects with the J. verreauxi enzyme, and that this enzyme is also inhibited by free Mg^{2+}, ADP^{3-} and ATP^{4-}. Therefore, high concentrations of these species were avoided in the experiments with the arginine kinase from P. longipes.

When purifying an enzyme for kinetic work, it is important to ensure that there are no contaminating enzyme activities which would interfere in the assays.

It is also of interest to establish whether the enzyme preparation has a high degree of homogeneity. Several methods are available for this, and the criteria of purity are based on the molecular size of the protein and on its charge.

For estimation of the homogeneity with regard to molecular weight, one of the most valuable techniques is column chromatography on Sephadex. Since proteins are separated on the basis of size by the molecular sieve properties of Sephadex, this method is particularly valuable in studying the homogeneity of an enzyme preparation, because both the activities, and protein concentrations in the peak fractions, can be quantitatively determined. Constancy of the specific activity of the enzyme in these fractions is a good indication of a high degree of purity.

The purity of a protein preparation may also be assessed electrophoretically, on the basis of the charge on the molecule. This can be achieved by starch gel electrophoresis, and there are a number of methods for detecting enzymic activity associated with a protein band. Polyacrylamide gel electrophoresis provides a more sensitive method for estimating homogeneity, since the molecular sieve properties of

the polyacrylamide gel result in separation of the components by molecular size as well as electrophoretic mobility.

In this chapter the purification of arginine kinase from P. longipes is described, and the degree of purity of the enzyme has been investigated. However, these studies were not exhaustive since the enzyme preparation appeared to be free of contaminating enzyme activities and was therefore suitable for kinetic work.

EXPERIMENTAL PROCEDURE

Materials

Phosphoarginine was isolated from crayfish muscle by the method of Marcus and Morrison (1964), and stored as the barium salt at -10°. Before use it was converted to the sodium salt by the method described by Uhr et al. (1966), and solutions were standardised by estimating the amount of arginine released after hydrolysis in 1 M HCl at 100° for 7 min (Rosenberg et al., 1956). ADP was obtained from P-L Biochemicals and used without further purification. ATP, also from P-L Biochemicals, was twice recrystallised by the method of Berger (1956). The purity of the nucleotides was checked by chromatography on DEAE-cellulose paper (Whatman paper, DE-20, from W. & R. Balston Ltd., England), as described by Morrison (1968). In each case, only a single spot absorbing ultraviolet light was detected. The concentrations of the solutions were checked by determining the absorption at 259 mμ in 0.01 M HCl (Bock et al., 1956). Triethanolamine (puriss) was obtained from Fluka A.-G., Buchs SG, Switzerland, and used without further purification, while N-ethylmorpholine from Eastman Organic Chemicals, Rochester, N.Y. was purified by distillation (Morrison

et al., 1961). Stock solutions of the aqueous buffers were prepared by weight. The pH of both these buffers was adjusted with 5 M HCl, using a Vibron pH meter and a constant temperature water bath, so that a 0.1 M solution was pH 8.0 at 30°. $MgCl_2.6H_2O$, from E. Merck A.-G., Darmstadt, was treated with dithizone, from British Drug Houses Ltd., as described by Morrison and Uhr (1966), and stock solutions were standardised by the method of Morrison et al. (1961). EDTA and bentonite were obtained from British Drug Houses, DEAE-cellulose (Cellex-D) from Bio-Rad Laboratories, Sephadex G-100 from Pharmacia, Uppsala, Sweden and hydrolysed starch from Connaught Laboratories, Toronto. Phosphoenolpyruvate, either as the monocyclohexylamine salt or the trisodium salt, was obtained from Calbiochem, as were crystalline preparations of muscle pyruvate kinase and lactic dehydrogenase.

Methods

Measurement of enzyme activity

The arginine kinase activity of the fractions obtained during purification was determined by measuring the initial rate of arginine release from phosphoarginine, under standard conditions. The reaction mixture (1.0 ml) contained 0.1 M N-ethyl-

morpholine-HCl buffer (pH 8.0), 0.1 mM EDTA, 2.5 mM phosphoarginine, 1.5 mM $MgCl_2$ and 0.625 mM ADP. The tubes were equilibrated at 30° in a water bath for at least 3 min, and the reaction was started by the addition of 5 µl of a suitably diluted enzyme solution. After a 3 min incubation, the reaction was stopped by the addition of 0.6 ml of 1.5 M NaOH, containing 0.14 M EDTA. The arginine formed was determined by measurement of the optical density at 535 mµ, 30 min after the addition of 1.0 ml of α-naphthol-diacetyl reagent (Rosenberg et al., 1956). Under these conditions, 0.1 µmole of arginine gave a reading of 0.770 in a cell of 1 cm light path. One unit of activity was taken as the release of 1 µmole of arginine per min, under the above conditions, and specific activity is defined as units per mg of protein.

Protein determination

Protein concentrations were estimated by the Biuret method of Gornall et al. (1949), with bovine serum albumin as the standard.

Ammonium sulphate fractionation

The weight (w) of ammonium sulphate necessary to give the desired degree of saturation was calculated using the formula :-

$$w = \frac{0.515\ V(S_2-S_1)}{1.0-0.292\ S_2}$$

where V represents the volume of the solution in millilitres, w is expressed in grammes and S_1 and S_2 are the initial and desired saturations with ammonium sulphate (0 to 1.0) at 0° (Kunitz, 1952; Noltmann et al., 1961).

ADP assay

ADP was estimated by a modification of the method of Bücher and Pfleiderer (1955). In the presence of NADH and phosphoenolpyruvate, NADH equivalent to the ADP present is oxidised to NAD^+, and this is measured by the decrease in extinction at 340 mμ when the reaction is complete. Under the conditions of assay, 0.1 μmole of ADP causes a decrease of 0.346 in the extinction, in a cell of 1 cm light path.

Chromatography on Sephadex G-100

Chromatography on Sephadex G-100 was carried out both at room temperature and at 4°, using a column (72.5 x 1.5 cm) equilibrated with 0.01 M N-ethylmorpholine-HCl buffer, pH 8.0, containing 0.1 mM EDTA. The enzyme was eluted with the same buffer at a flow rate of approximately 0.2 ml per min. 1.8 ml fractions were collected.

Electrophoresis

(a) Starch gel electrophoresis. Starch gel electrophoresis was carried out according to the method of Smithies (1955) using a horizontal gel of dimensions 20 x 6 x 0.4 cm. Hydrolysed starch was used and the gels were prepared with starch concentrations 1% higher than those recommended by the manufacturer. Gels were prepared in 0.05 M Tris-HCl buffer, pH 8.7, and the electrode vessels contained the same buffer at a concentration of 0.125 M. Electrophoresis was carried out for 12 hr at 20 V/cm at an ambient temperature of 4°.

After electrophoresis, the gels were sliced horizontally and treated in the following way :

(i) On one half of the gel protein was fixed and stained with a 1% solution of amido black in methanol/acetic acid/water (50:20:50, v/v) and excess stain removed by washing with the same solvent until the background was pale blue.

(ii) Activity was detected on the other half of the gel by soaking a sheet of filter paper with the reaction mixture containing phosphoarginine and $MgADP^-$ (p. 36), removing excess moisture, and laying the paper on the gel. After incubat-

ing at room temperature for about 10 min the paper was allowed to dry in air and sprayed with a 1:1 solution of 2 M NaOH and α-naphthol-diacetyl reagent, which were mixed immediately before use. A pink band was visible at the position of the major protein band.

(b) Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the purified enzyme preparation was carried out as described by Davis (1964), except that the stacking gel consisted of 2.5% acrylamide and 0.3% bis-acrylamide, and the resolving gel consisted of 9% acrylamide and 0.1% bis-acrylamide. Also, electrophoresis was carried out at 4°, instead of at room temperature.

RESULTS

Arginine kinase was purified from the tail muscle of P. longipes by a method similar to that previously described by Uhr et al. (1966) for the purification of arginine kinase from J. verreauxi. The crayfish were collected and the shells immediately removed from the tail muscles, which were then frozen in liquid nitrogen and subsequently stored at -15°. The arginine kinase activity of the muscle remained constant for at least a year under these conditions.

Extraction of muscle

100 grammes of muscle was weighed out and cut into small pieces, which were allowed to thaw at room temperature. All subsequent steps of the purification procedure were carried out at 4°.

The muscle was stirred in 250 ml of 1.3 mM EDTA (pH 7.0) for 15 min. This mixture was homogenised for 1 min in a Waring Blendor and the suspension centrifuged at 3,000 x g. for 20 min. The cloudy supernatant was retained and the residue re-suspended in 100 ml of the EDTA solution, homogenised for 30 sec and centrifuged as above. The supernatants from the two centrifugations were combined and the opalescent solution was immediately adjusted to pH 8.0 by the addition of 5 M NaOH.

Bentonite treatment of extract

The total protein in the muscle extract was determined and 2 grammes of bentonite per gramme of protein was added, with very efficient stirring, over a period of 5 min. After stirring for a further 5 min, the solution was centrifuged at 3,000 x g. for 20 min and the clear supernatant retained. 0.125 vol. of 0.5 M N-ethylmorpholine-HCl buffer, pH 8.0, was added to this solution.

Ammonium sulphate fractionation

Finely ground ammonium sulphate was added to the solution, with stirring, over a period of about 1 hr, to bring it to 0.6 saturation. After standing for 30 min, the solution was centrifuged at 4,000 x g. for 20 min and the precipitate discarded. The clear supernatant was brought to 0.85 saturation by addition, with stirring, of finely ground ammonium sulphate over approximately 30 min and, after standing for 1 hr, the precipitate was collected by centrifuging and the supernatant discarded. The precipitate was suspended in 15 ml of 0.01 M N-ethylmorpholine-HCl buffer containing 1.0 mM EDTA (pH 8.0), and dialysed against three changes, each of 2 litres, of the same buffer, over a period of at least 17 hr. The precipitate was

then removed by centrifuging, as above, and the clear supernatant retained.

Chromatography on DEAE-cellulose

The clear solution from the previous step (approximately 35 ml) was applied to a DEAE-cellulose column (2 x 45 cm), and washed into the column with 30 ml of 0.01 M N-ethylmorpholine-HCl buffer (pH 8.0) containing 1.0 mM EDTA. The enzyme was then eluted with a linear gradient, prepared from 300 ml of this buffer and 300 ml of 0.1 M N-ethylmorpholine-HCl (pH 8.0) containing 1.0 mM EDTA. The fractions with the highest specific activity were pooled and dialysed against three changes, each of one litre, of the same buffer as used in the previous dialysis step, for 17 hr. The dialysate was applied to a second DEAE-cellulose column, of the same dimensions as above, and, after washing the enzyme solution into the column as described previously, a linear gradient of 300 ml of the dialysis buffer and 300 ml of 0.05 M N-ethylmorpholine-HCl containing 1.0 mM EDTA was applied. Again the fractions with highest specific activity were pooled and dialysed against two changes (1 litre each) of the dialysis buffer. If the protein concentration of the resulting solution was less than 6 mg/ml,

it was concentrated using a Diaflo apparatus. A summary of yields and specific activities obtained during a typical purification is given in Table I.1. The range of specific activities of arginine kinase preparations varied from approximately 160 to 200 units per mg of protein. However, it is difficult to determine the specific activity of a preparation accurately, because the assay is so sensitive that an enzyme solution containing 6 to 12 mg/ml has to be diluted 200,000 times before the activity can be estimated.

Stability of purified arginine kinase

The purified enzyme was stored at 4°, in 0.01 M N-ethylmorpholine-HCl buffer (pH 8.0), at protein concentrations of 6 to 12 mg/ml and, under these conditions, was stable for at least a year. Slight precipitation of the protein occurred, but the specific activity of the enzyme did not change when the precipitate was removed by centrifugation. The enzyme was stable for at least 3 hr at 0° in the presence of 0.001 M triethanolamine (pH 8.0) at a concentration of approximately 0.1 mg/ml. However, freezing of the enzyme resulted in precipitation of much of the protein, with marked loss of activity.

Table I.1.

Summary of yields and specific activities of fractions obtained during the purification of arginine kinase from the tail muscle of the crayfish, Panulirus longipes.

Details are given in the text. Weight of muscle, 100 grammes.

Fraction	Volume	Protein	Total Units	Specific Activity
	ml	mg		units/mg
Extract	318	5,931	249,000	42
Supernatant after bentonite treatment	315	3,355	198,450	59
Dialysed ammonium sulphate precipitate (0.60-0.85 saturation)	37.5	1,950	163,460	84
Dialysed eluate from first DEAE-cellulose column	31.6	493	58,780	119
Dialysed eluate from second DEAE-cellulose column	29.5	242	38,910	161

Contaminating enzyme activities

The enzyme preparations were tested for ATPase, myokinase and phosphoamidase activities, as described by Morrison et al. (1957), using concentrations which were approximately 100-fold greater than those used in kinetic experiments. No myokinase or phosphoamidase activities could be detected. However, there appeared to be some ATPase activity, but the ADP formed did not increase with the time of incubation. Thus, it was suspected that the apparent ATPase activity was due to the presence of ADP in the ATP solution. Approximately 0.1 μmole of arginine was released when the enzyme was incubated with phosphoarginine (5 mM) and ATP (10 mM), and direct assay of the ATP for ADP, as described in the Methods section, confirmed that the ATP contained approximately 1% ADP. Because the arginine kinase preparations appeared to be free of enzymic activities which would interfere with kinetic studies on the reaction mechanism, they were considered suitable for such studies.

Chromatography on Sephadex G-100

Chromatography of a sample of the purified enzyme, on a Sephadex G-100 column (72 x 1.5 cm) at room temperature, resulted in a single symmetrical peak with

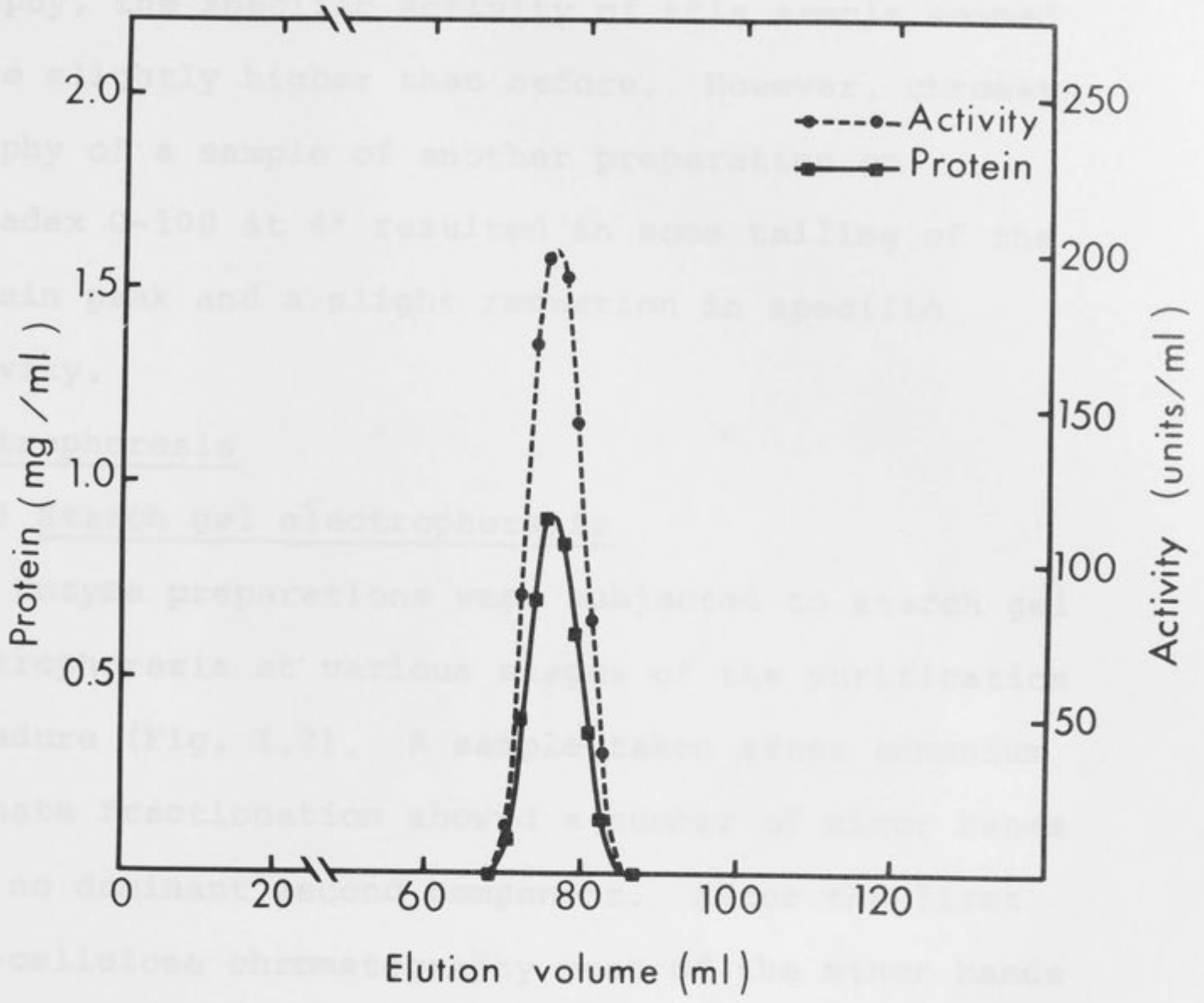


Fig. I.1. Chromatography of a sample of purified arginine kinase on Sephadex G-100 at room temperature.

constant specific activity (Fig. I.1). After chromatography, the specific activity of this sample seemed to be slightly higher than before. However, chromatography of a sample of another preparation on Sephadex G-100 at 4° resulted in some tailing of the protein peak and a slight reduction in specific activity.

Electrophoresis

(a) Starch gel electrophoresis

Enzyme preparations were subjected to starch gel electrophoresis at various stages of the purification procedure (Fig. I.2). A sample taken after ammonium sulphate fractionation showed a number of minor bands with no dominant second component. After the first DEAE-cellulose chromatography most of the minor bands could not be detected, and samples of the final preparation showed only one protein component. Enzymic activity was shown to be associated with this band of protein.

It is possible that, during purification, the minor bands could have been decreased but not completely eliminated. Starch gel electrophoresis would not be sensitive enough to allow detection of a number of very minor contaminants.

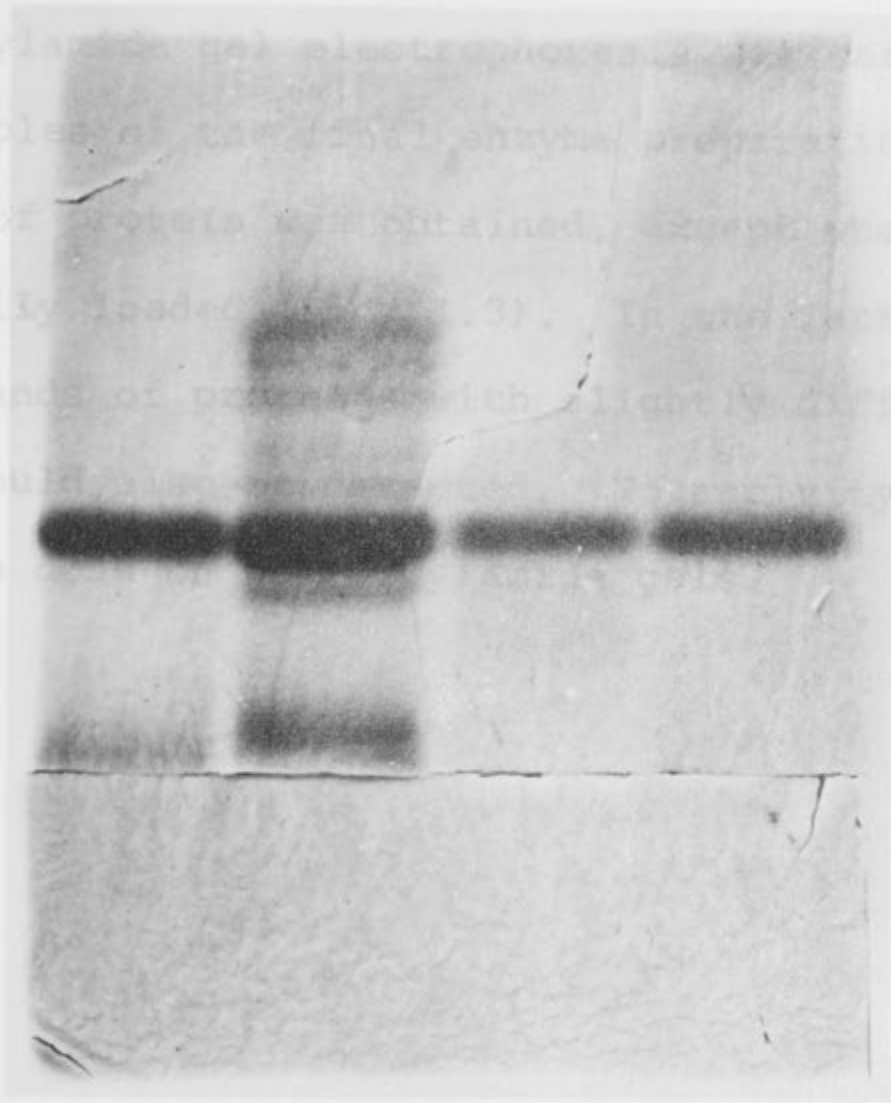


Fig. I.2. Samples of arginine kinase stained for protein after starch gel electrophoresis. A, 100 μg sample after chromatography on first DEAE-cellulose column; B, dialysed sample (400 μg) of ammonium sulphate precipitate (0.60-0.85 saturation) after centrifugation; C, and D, 50 μg samples of two different arginine kinase preparations after the final stage of purification.

(b) Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out with samples of the final enzyme preparation. A single band of protein was obtained, except when the gel was heavily loaded (Fig. I.3). In the latter case very minor bands of proteins with slightly different mobilities could also be detected. No activity determinations were done on polyacrylamide gels.

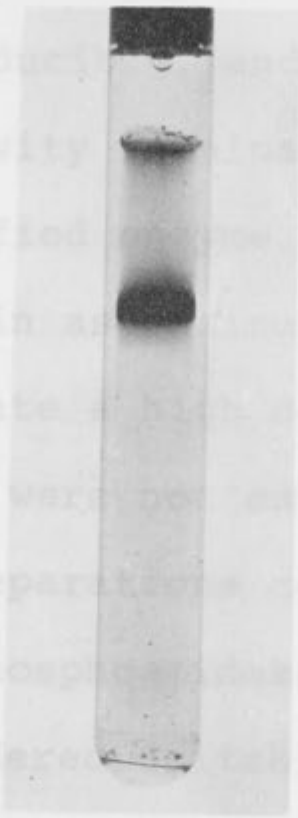


Fig. I.3. A sample of purified arginine kinase (50 µg) stained for protein after polyacrylamide gel electrophoresis.

The polyacrylamide gel electrophoresis was carried out by Miss J.M. La Nauze.

DISCUSSION

The method described for the purification of arginine kinase from the tail muscle of P. longipes has been shown to be reproducible, and it has been observed that the specific activity remains constant for long periods, when the purified enzyme is stored at 4°.

The methods used in assessing the homogeneity of the preparations indicate a high degree of purity, although these studies were not exhaustive. The purified arginine kinase preparations contained no detectable ATPase, myokinase or phosphoamidase activities and were, therefore, considered suitable for kinetic studies. In addition, electrophoresis on starch and polyacrylamide gels indicated a high degree of homogeneity. From the low mobility of the protein on polyacrylamide gels, and because of the low ionic strengths of buffer required to elute the enzyme from DEAE-cellulose, it appears that the molecule has a relatively low surface charge.

Although there was a slight increase in the specific activity of one enzyme preparation after chromatography on Sephadex G-100 at room temperature, the same procedure carried out at 4° with another preparation resulted in a slight decrease in specific

activity. The reason for these differences is not apparent but, in general, chromatography on Sephadex G-100 does not appear to affect the specific activity of the enzyme to any great extent, and was not, therefore, included as a step in the purification procedure.

The purification procedure used for the arginine kinase from P. longipes is very similar to that used for this enzyme from J. verreauxi (Uhr et al., 1966), and also resembles that of Virden et al. (1965) for the arginine kinase from H. vulgaris. Blethen and Kaplan (1967), however, obtained crystalline arginine kinase from H. americanus by repeated ammonium sulphate fractionation of crude muscle extracts. This preparation appeared homogeneous on starch gel electrophoresis, and a single precipitin line was obtained on immunodiffusion in agar gel. Attempts to crystallise the arginine kinase from P. longipes, using ammonium sulphate at room temperature, were not successful, since the enzyme appeared to form an amorphous precipitate rather than to crystallise.

SUMMARY

1. Arginine kinase has been purified, by a reproducible method, from the tail muscle of P. longipes.

2. The enzyme has been found to be stable for at least a year under suitable conditions.

3. The preparations appear to have a high degree of homogeneity, as judged by starch and polyacrylamide gel electrophoresis and chromatography on Sephadex G-100.

4. The purified arginine kinase preparations are free of any contaminating ATPase, myokinase or phosphoamidase activities which would interfere in kinetic studies.

CHAPTER II

Initial Velocity and Isotope Exchange Studies with Arginine Kinase from P. longipes

INTRODUCTION

The reaction mechanism of creatine kinase from rabbit muscle has been extensively investigated and has been found to be rapid equilibrium, random, with two dead-end complexes (Kuby and Noltmann, 1962; Morrison and James, 1965; Morrison and Cleland, 1966; James and Morrison, 1966a, 1966b; O'Sullivan and Cohn, 1966a). The nature of the reaction mechanism of arginine kinase, on the other hand, has not been studied in such detail, and no reaction mechanism has been definitely established. The fact that several types of muscle have been used as sources of arginine kinase has complicated the study of the enzyme, since the results obtained by various groups of workers cannot be compared directly.

The first initial velocity studies with arginine kinase were carried out by Virden et al. (1965), using the enzyme purified from the tail muscle of the European lobster, H. vulgaris. The double reciprocal plots of initial velocity against substrate concentration obtained for the forward direction of the reaction, with $MgATP^{2-}$ and arginine as substrates, consisted of families of straight lines intersecting to the left of the vertical axis. These results

indicate that the enzyme has a sequential mechanism, but since no further kinetic studies were undertaken, the mechanism was not defined.

The reaction mechanism of the arginine kinase from the Australia sea-water crayfish, J. verreauxi, has been investigated by initial velocity studies in this laboratory, and families of parallel lines were obtained in double reciprocal plots for both directions of the reaction (Uhr et al., 1966). This suggested that the reaction mechanism was ping pong, with one substrate reacting with the enzyme and one product released before the second substrate adds to the enzyme. Partial exchange reactions consistent with such a mechanism were also demonstrated, both between arginine and phosphoarginine, and between $MgADP^-$ and $MgATP^{2-}$. Quantitative determinations of the maximum rates of isotope exchange, however, showed that these were 500 to 1000 times lower than those calculated using the kinetic constants obtained from initial velocity and product inhibition data (Uhr, personal communication). These results cast doubt on the conclusion that the reaction mechanism is ping pong.

In the hope of reaching a more definite conclusion about the reaction mechanism of another arginine kinase, it was decided to make a detailed kinetic study of the enzyme from a different organism. The source chosen was the tail muscle of the West Australian sea-water crayfish, P. longipes, and the results of initial velocity and isotope exchange studies are reported in this chapter.

EXPERIMENTAL PROCEDURE

Materials

L-Arginine (monohydrochloride) was obtained from British Drug Houses Ltd., and, after adjustment to pH 8.0 with 1 M HCl, solutions were stored at -10°. AMP was obtained from P-L Biochemicals. $MnCl_2.4H_2O$ and $CaCl_2.6H_2O$ were Analar grade from British Drug Houses Ltd., and, after treatment with dithizone, solutions were standardised by the method of Morrison et al. (1961). Trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (DCTA) was from Fluka.

[8-^{14}C]ADP-Li_3 (38.6 mC per mmole), [8-^{14}C]ATP-Li_4 (28 mC per mmole) and [^{14}C]L-arginine (182 mC per mmole) were purchased from Schwarz BioResearch, Inc. The samples of radioactive nucleotides were in 50% ethanol and were evaporated to dryness in a stream of dry air and redissolved in water before use, since ethanol may inactivate the enzyme (Morrison et al., 1957; Virden et al., 1965). All other chemicals were as described in Chapter I. Arginine kinase was prepared by the method given in Chapter I.

Methods

Measurement of initial velocities

Detailed kinetic studies were carried out at 30° in the presence of 0.1 M triethanolamine-HCl buffer

(pH 8.0) containing 0.01 mM EDTA, with enzyme concentrations of approximately 0.2 µg/ml. After the addition of the components of the reaction mixture and before the addition of enzyme, the tubes were incubated for at least 3 min at 30°. The enzyme was diluted with 0.001 M triethanolamine-HCl buffer (pH 8.0) and 2 µl was added to each tube to start the reaction. For the addition of enzyme a Hamilton microsyringe (100 µl) fitted with a Chaney adaption, which delivers one-fiftieth of the syringe volume per delivery, was used. In all cases the reactions were run for at least two time periods to ensure that initial velocities were being measured. Extinction measurements were made using either a Shimadzu or a Gilford 300 spectrophotometer.

Mg^{2+} was normally used as the activating metal ion and, under the experimental conditions, the reaction was assumed to be :-

$$MgATP^{2-} + \text{arginine} \rightleftharpoons MgADP^{-} + \text{phosphoarginine}$$

Thus, the Mg-nucleotide complexes and the free forms of arginine and phosphoarginine were assumed to be the substrates. The concentration of free Mg^{2+} was held constant at 1.0 mM, and, as in previous kinetic

studies with phosphagen phosphotransferases (Morrison and James, 1965; Uhr et al., 1966), the concentrations of total metal and total substrates required to give the desired concentrations of the various ionic species were calculated (Morrison et al., 1961). The stability constants for $MgATP^{2-}$, $MgADP^{-}$, Mg-phosphoarginine and Mg-arginine were taken to be 70,000 M^{-1}, 4,000 M^{-1}, 100 M^{-1} and 20 M^{-1}, respectively and those for $MnADP^{-}$ and $CaADP^{-}$, 2,200 M^{-1} and 25,000 M^{-1} (O'Sullivan and Perrin, 1964; Pelletier, 1964). The stability constants for Mn-phosphoarginine and Ca-phosphoarginine were assumed to be the same as those for the Mg-phosphoarginine complex, and that for MgAMP to be 89 M^{-1} (Walaas, 1958). Free AMP was taken to be the inhibitory species.

Arginine and ADP were assayed as described in Chapter I except that, when Mn^{2+} was the activating ion, the arginine assay was modified to avoid precipitation (Morrison and Uhr, 1966). The reaction in the presence of Mn^{2+} was stopped by the addition of 0.5 ml DCTA (0.081 M, pH 12.0), and 0.3 ml of 3 M NaOH was added 5 sec before the α-naphthol diacetyl reagent. ADP was assayed in studies of the forward direction of the reaction, and arginine in studies on the reverse reaction.

Determination of isotope exchange rates

(a) Partial isotope exchanges. The experimental conditions for the partial exchange reactions are given in the legend to Fig. II.3. The general procedures used were similar to those described by Morrison and Cleland (1966) and Morrison and White (1967), and the exchange reactions were stopped by application of samples of the reaction mixture (20 to 40 µl) to DEAE-cellulose paper. The nucleotides were then separated by ascending chromatography, using the method of Morrison (1968). This involves chromatography for 4 hr with 0.6 M ammonium formate at pH 3.1. Under these conditions the nucleotides have different mobilities, and the positions of the spots after chromatography can easily be detected by absorption of ultraviolet light. The guanidino reactants were separated by ascending chromatography on DEAE-cellulose paper with pyridine/NH_3 (specific gravity 0.88)/water (60:15:15, v/v) for 16 hr at room temperature. This resulted in good separation of arginine and phospho-arginine, with the latter remaining at, or very near, the origin. Arginine was detected by spraying the chromatogram with a solution containing equal volumes of 2 M NaOH and the α-naphthol diacetyl reagent

(Rosenberg et al., 1956), which were mixed immediately before use. Arginine appeared as a pink spot. The position of phosphoarginine could also be determined by suspending the chromatogram in a closed vessel above concentrated HCl for 1 hr to hydrolyse the phosphoarginine to arginine. After removing the HCl by hanging the paper in a stream of air, arginine could be detected as described above.

These treatments of the paper did not appear to cause any quenching, and the spots were cut out and counted, using a Packard Tri-Carb scintillation counter, as described by Morrison and Cleland (1966). From the results, the initial rates of isotope exchange between the nucleotides were calculated.

(b) Isotope exchange at equilibrium. In the studies of isotope exchange at equilibrium the methods used were similar to those described above, except that all reactants were present and equilibrium conditions were established enzymically before the addition of labelled reactants. The conditions under which isotope exchange studies at equilibrium were carried out are given in the legend to Table II.3 and in Table II.4.

Determination of equilibrium constant for reaction

The conditions under which the equilibrium constant was determined are given in the legend to Table II.3. In Experiment I, 0.3 ml samples were taken and the reaction stopped at intervals between 8 and 14 min by the addition of 5.0 ml of 0.081 M DCTA (pH 12.0). A 1.7 ml aliquot of each of these samples was then taken for arginine assay. In Experiments II and III, 5.0 ml of 0.081 M DCTA (pH 12.0) was added to each 0.3 ml sample at intervals between 16 and 24 min, and 20 and 35 min, respectively. No further additions were made to samples from Experiment II, and 4.7 ml of water was added to each sample from Experiment III. 0.7 ml aliquots were then taken from each sample for arginine assay.

The total concentrations of ATP, ADP and phospho-arginine present at equilibrium were calculated from the equilibrium concentrations of arginine under each set of conditions. The equilibrium concentrations of the Mg-complexes of the reactants were calculated, using the NUCALC computer programme in conjunction with an IBM 360 computer, and in these calculations the concentrations of the Mg-arginine complex were considered negligible.

Analysis of kinetic data

All initial velocity data were plotted in double reciprocal form and those experimental points which appeared inconsistent with the general pattern were discarded. Analysis of the remaining experimental data was carried out using the appropriate computer programme of Cleland (1963d) in conjunction with an IBM 360 computer. Initial velocity data were fitted using the HYPER, LINE, PARA, PING PONG and SEQUEN programmes (Preface; Equations 1, 2, 3, 4 and 5). The values of the kinetic constants and their standard errors were obtained from the computer output. The weighted mean values of constants, together with their standard errors (S.E.), were calculated using the formulae :-

$$\text{Weighted mean of X values} = \frac{\Sigma W_i X_i}{\Sigma W_i}$$

$$\text{S.E. of weighted mean value} = \frac{1}{\sqrt{\Sigma W_i}}$$

$$\text{where } W_i = \frac{1}{[\text{S.E.}(X_i)]^2}$$

The standard error (σ) of the product of constants with standard errors was calculated using the relation-

ship :-

$$\sigma(xy) = xy\sqrt{\left[\frac{S.E.(x)}{x}\right]^2 + \left[\frac{S.E.(y)}{y}\right]^2}$$

while that for the quotient was obtained from the relationship :-

$$\sigma\left(\frac{x}{y}\right) = \frac{x}{y}\sqrt{\left[\frac{S.E.(x)}{x}\right]^2 + \left[\frac{S.E.(y)}{y}\right]^2}$$

In all plots the points are those obtained experimentally while the lines are drawn using the kinetic constants obtained from computer analysis of the data.

THEORY

As discussed in the general introduction, there are four basic types of reaction mechanism for bi-reactant enzymes such as arginine kinase, which catalyses the reaction :-

$$\text{arginine} + \text{ATP}^{4-} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{phosphoarginine} + \text{ADP}^{3-}$$

Of the possible basic mechanisms, one is non-sequential, or ping pong, and the other three are sequential (p. 6).

Ping pong mechanism

The initial velocity equation for a ping pong mechanism is :-

$$v = \frac{V_1 AB}{K_a B + K_b A + AB} \qquad \text{(II.1)}$$

where v is the initial velocity, V_1 is the maximum velocity in the forward direction, A and B are the substrate concentrations and K_a and K_b are their respective Michaelis constants. When rearranged in double reciprocal form this equation becomes :-

$$\frac{1}{v} = \frac{K_a}{V_1}\cdot\frac{1}{A} + \frac{1}{V_1}\left(\frac{K_b}{B} + 1\right) \qquad \text{(II.2)}$$

Thus a double reciprocal plot of v against A gives a series of parallel lines, whose intercepts on the $\frac{1}{v}$ axis are dependent on the concentration of the fixed substrate, B. When the substrates, A and B, are held in constant ratio, (B = xA), equation II.2 can be written :-

$$\frac{1}{v} = \left\{ \frac{K_b}{x} + K_a \right\} \frac{1}{A} + \frac{1}{V_1} \qquad \text{(II.3)}$$

This is the equation for a straight line.

Sequential mechanism

For a sequential mechanism the initial velocity equation is :-

$$v = \frac{V_1 AB}{K_{ia} K_b + K_a B + K_b A + AB} \qquad \text{(II.4)}$$

The meaning of the kinetic constants in this equation depends on the actual reaction mechanism of the enzyme. In double reciprocal form this equation can be written :-

$$\frac{1}{v} = \frac{1}{V_1} \left\{ \frac{K_{ia} K_b}{B} + K_a \right\} \frac{1}{A} + \frac{1}{V_1} \left\{ \frac{K_b}{B} + 1 \right\} \qquad \text{(II.5)}$$

so that both the slopes and the intercepts of the lines in a double reciprocal plot of v against A vary

with the concentration of B, and a family of intersecting lines is obtained. When the substrates are held in constant ratio, (B = xA), the equation for a sequential reaction can be written :-

$$\frac{1}{v} = \frac{1}{V_1}\left\{\frac{K_{ib}K_a}{x}\right\}\frac{1}{A^2} + \frac{1}{V_1}\left\{\frac{K_b}{x} + K_a\right\}\frac{1}{A} + \frac{1}{V_1} \qquad \text{(II.6a)}$$

A double reciprocal plot of v against A then has the form of a parabola.

RESULTS

Initial velocity studies

Initial velocity studies with arginine kinase were carried out in both directions of the reaction, as described in the Methods section. The patterns obtained for the forward and reverse reactions are shown in Fig. II.1 in double reciprocal form, and it appears that the changes in slope of the lines are small or negligible when the concentration of the fixed substrate is changed, indicating that the reaction mechanism may be ping pong. In the reverse direction of the reaction, substrate inhibition of the reaction occurs at concentrations of phosphoarginine above 10 mM, and therefore the highest concentration of this substrate used in initial velocity studies was 5 mM.

Substrate inhibition by $MgADP^-$ was also observed at concentrations above 1 mM, but this may have been due, in part at least, to the free ADP^{3-} present at these concentrations.

The data from these initial velocity studies were analysed using the PING PONG computer programme, and the values for Michaelis constants obtained in this way are given in Table II.1.

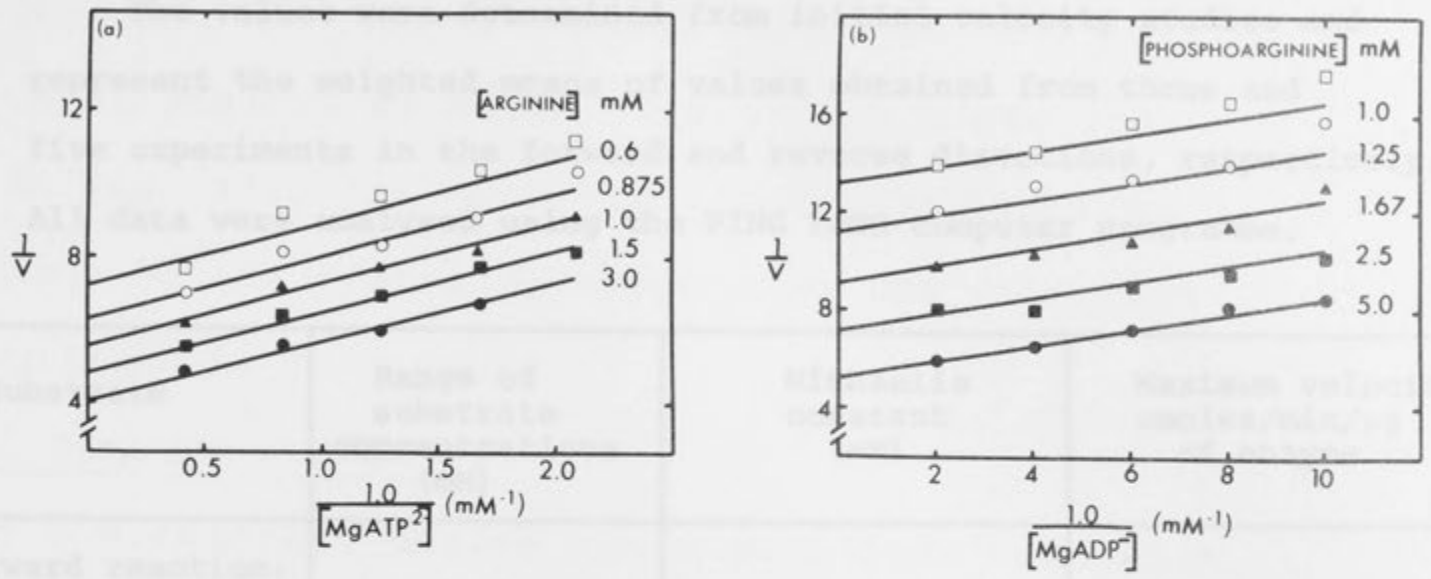


Fig. II.1. (a) Effect of arginine on the initial velocity of the forward reaction with $MgATP^{2-}$ as the variable substrate. (b) Effect of phosphoarginine on the initial velocity of the reverse reaction with $MgADP^{-}$ as the variable substrate. Velocities are expressed as μmoles per min per μg of arginine kinase.

Table II.1.

Values for Michaelis constants and maximum velocities for the forward and reverse directions of the arginine kinase reaction.

The values were determined from initial velocity studies and represent the weighted means of values obtained from three and five experiments in the forward and reverse directions, respectively. All data were analysed using the PING PONG computer programme.

Substrate	Range of substrate concentrations (mM)	Michaelis constant (mM)	Maximum velocity µmoles/min/µg of enzyme
Forward reaction:			
$MgATP^{2-}$	0.48 → 2.4	0.57 ± 0.03	0.331 ± 0.008
Arginine	0.6 → 3.0	0.82 ± 0.04	
Reverse reaction:			
Phosphoarginine	1.0 → 5.0	3.04 ± 0.13	0.303 ± 0.009
$MgADP^{-}$	0.1 → 0.5	0.112 ± 0.007	

As discussed in the Theory section, when the reaction mechanism of an enzyme is ping pong a double reciprocal plot of initial velocity against substrate concentration, with the substrates held in constant ratio, must yield a straight line. Such plots are shown in Fig. II.2 for both the forward and reverse directions of the reaction, supporting the view that the mechanism is ping pong.

Partial isotope exchanges

If a bireactant enzyme has a ping pong mechanism, it should be possible to demonstrate a partial exchange reaction between an appropriate pair of reactants in the absence of the other pair. These partial exchanges were shown to occur both between arginine and phospho-arginine and between ADP and ATP, in the presence of the metal ions, Mg^{2+}, Mn^{2+} and Ca^{2+} (Fig. II.3). The latter exchange is completely dependent on the presence of a metal ion, while the arginine-phospho-arginine exchange proceeds in the absence of metal ions, is activated by Mg^{2+}, and is inhibited by Ca^{2+} and Mn^{2+}. (The concentration of free Mn^{2+} was reduced to 0.5 mM because of the marked inhibition of the exchange reaction with this metal ion present at 2.0 mM). The relative effects of these metal ions on the rates

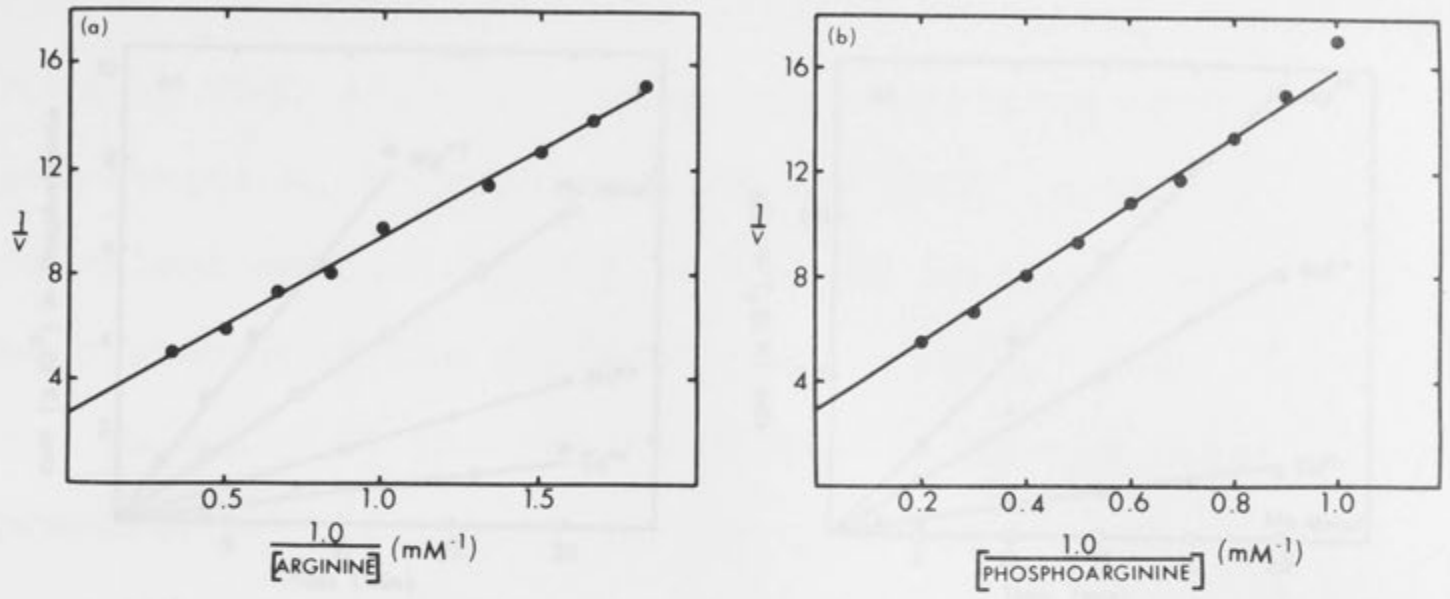


Fig. II.2. Effect on the initial velocity of the arginine kinase reaction of varying the concentrations of the substrates in constant ratio : (a) forward reaction with $MgATP^{2-}$:arginine = 0.8:1.0; (b) reverse reaction with phosphoarginine:MgADP = 10:1. Velocities are expressed as μmoles per min per μg of arginine kinase.

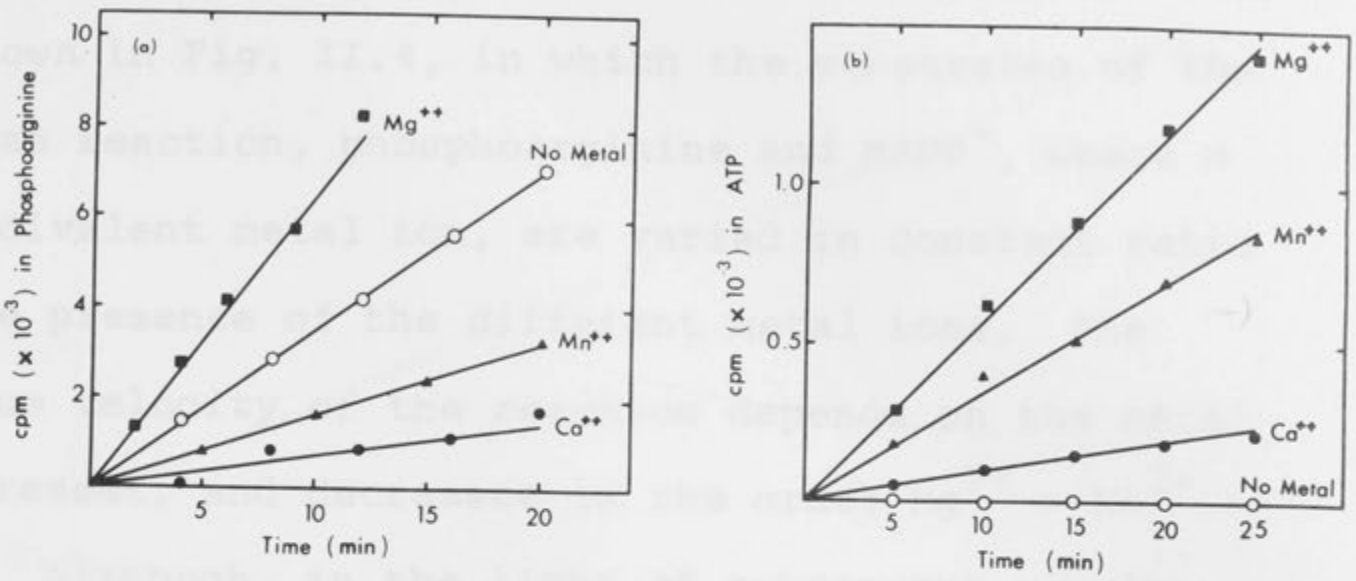


Fig. II.3. (a) Effect of divalent metal ions on initial rate of incorporation of label from [^{14}C]L-arginine into phosphoarginine in the absence of nucleotide substrates. Reaction mixtures contained in a final volume of 0.25 ml : triethanolamine-HCl buffer (pH 8.0), 0.1 M; EDTA, 0.1 mM; phosphoarginine, 1.0 mM; [^{14}C]L-arginine, 0.45 μC (0.25 mμmole); arginine kinase, 36 μg; and where indicated Mg^{2+}, 2.0 mM; Mn^{2+}, 0.5 mM or Ca^{2+}, 2.0 mM.

(b) Effect of divalent metal ions on initial rate of incorporation of label from [^{14}C]ADP into ATP in the absence of guanidino substrates. Reaction mixtures (0.25 ml) contained the same concentrations of buffer and EDTA as in (a); ATP, 3.0 mM; ADP, 0.5 mM; [^{14}C]ADP, 0.17 μC (4.4 mμmoles); arginine kinase, 18 μg; and divalent metal ion, 2.0 mM. The concentrations of metal ion given represent free concentrations.

of isotope exchange were found to be similar to their effects on the velocity of the overall reaction. This is shown in Fig. II.4, in which the substrates of the reverse reaction, phosphoarginine and $MADP^-$, where M is a divalent metal ion, are varied in constant ratio in the presence of the different metal ions. The maximum velocity of the reaction depends on the metal ion present, and decreases in the order $Mg^{2+} > Mn^{2+} >> Ca^{2+}$. Although, in the light of subsequent results, these maximum velocities cannot be considered accurate, the relative magnitudes of the values would be correct.

The results given above are qualitatively consistent with a ping pong mechanism for the reaction, and it is unlikely that both exchanges would occur, and be affected by metal ions in a similar way to the overall arginine kinase reaction, if the partial reactions were not catalysed by this enzyme. However, from the data in Fig. II.4, the initial rates of the ADP-ATP exchange were calculated to be 12.4, 7.5 and 1.9 μmoles per min per μg of enzyme, in the presence of Mg^{2+}, Mn^{2+} and Ca^{2+}, respectively. These rates appeared to be at least two orders of magnitude lower than would be expected on the basis of observations of the initial rate of the overall reaction. If the

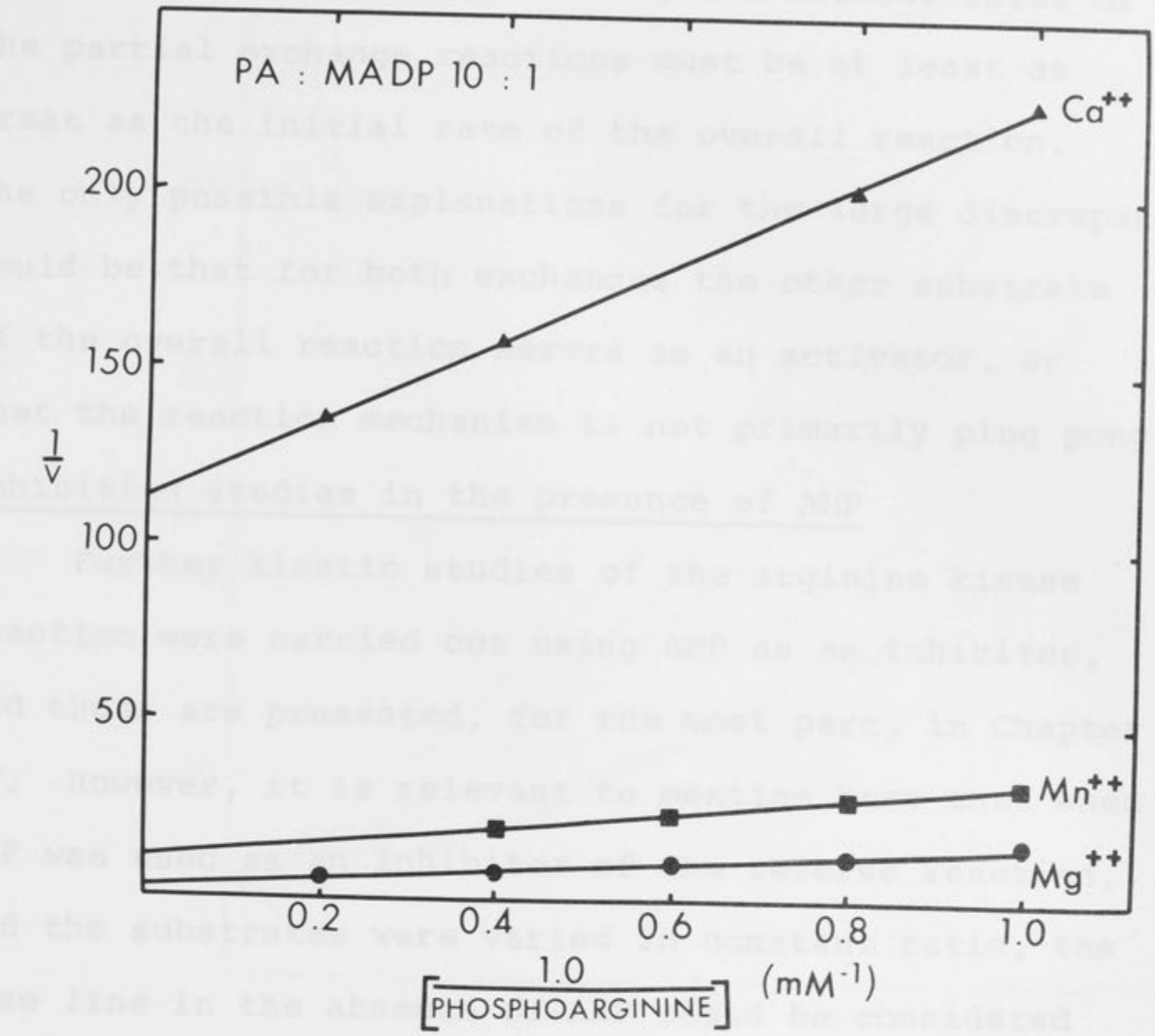


Fig. II.4. Determination of the maximum velocity of the arginine kinase reaction, in the presence of different metal ions, by simultaneously varying the concentrations of phosphoarginine and $MgADP^-$ in a constant ratio of 10:1. The concentration of free metal ion was maintained at 2.0 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.

mechanism is ping pong, however, the initial rates of the partial exchange reactions must be at least as great as the initial rate of the overall reaction. The only possible explanations for the large discrepancy would be that for both exchanges the other substrate of the overall reaction serves as an activator, or that the reaction mechanism is not primarily ping pong.

Inhibition studies in the presence of AMP

Further kinetic studies of the arginine kinase reaction were carried out using AMP as an inhibitor, and these are presented, for the most part, in Chapter IV. However, it is relevant to mention here that when AMP was used as an inhibitor of the reverse reaction, and the substrates were varied in constant ratio, the base line in the absence of AMP could be considered linear, but increasing the concentration of AMP caused the lines to become increasingly parabolic (Fig. II.5). All the lines gave a good fit to the equation for a parabola, and the base line also gave a good fit to the equation for a straight line. These results are not consistent with the enzyme having a ping pong mechanism, and thus the mechanism must be sequential. Therefore, further initial velocity studies were carried out over lower ranges of substrate concentrations, to determine whether it was possible to obtain

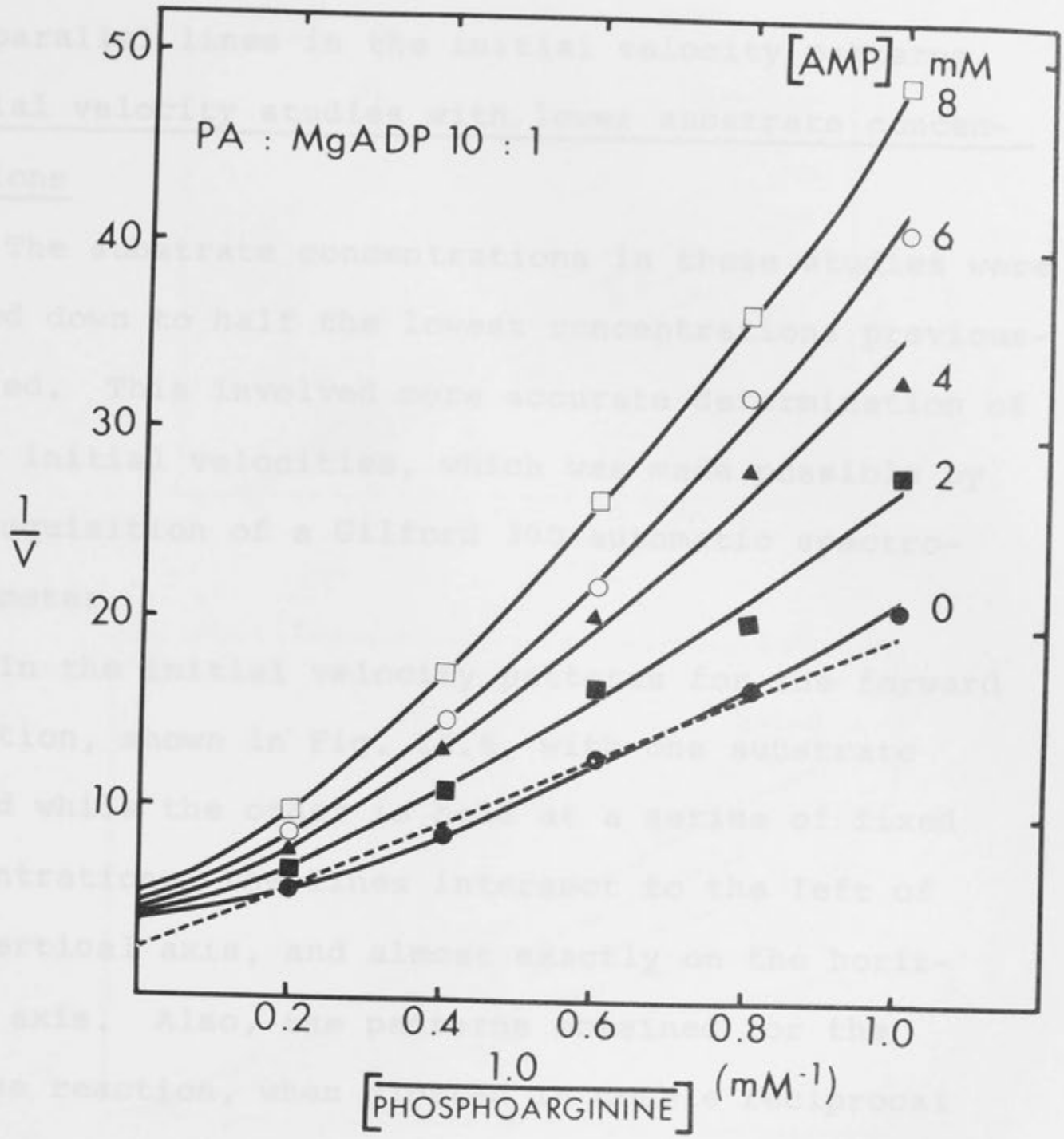


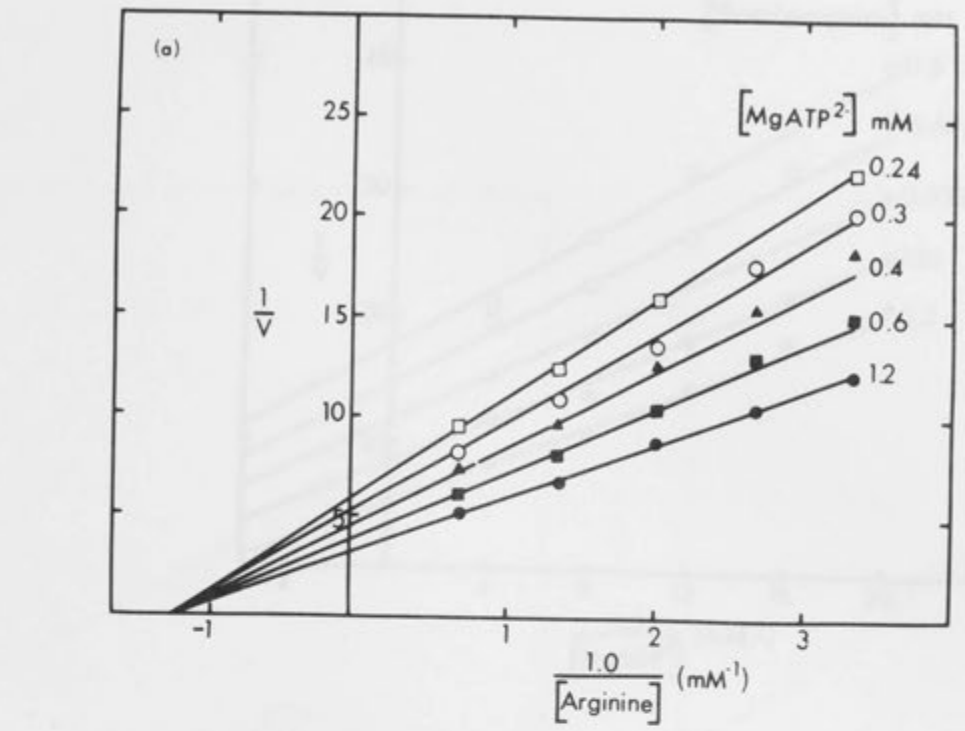
Fig. II.5. Inhibition of the reverse reaction by AMP under conditions where phosphoarginine and $MgADP^-$ were varied in a constant ratio of 10:1. The solid lines were drawn using the constants obtained by fitting the data using the PARA computer programme. The broken line shows the fit obtained when the initial velocity data, in the absence of AMP, were fitted using the LINE programme. Velocities are expressed as µmoles per min per µg of arginine kinase.

non-parallel lines in the initial velocity patterns.

Initial velocity studies with lower substrate concentrations

The substrate concentrations in these studies were varied down to half the lowest concentrations previously used. This involved more accurate determination of lower initial velocities, which was made possible by the acquisition of a Gilford 300 automatic spectrophotometer.

In the initial velocity patterns for the forward direction, shown in Fig. II.6, with one substrate varied while the other is held at a series of fixed concentrations, the lines intersect to the left of the vertical axis, and almost exactly on the horizontal axis. Also, the patterns obtained for the reverse reaction, when plotted in double reciprocal form (Fig. II.7), show some change in slope with decreasing concentrations of fixed substrate. For both directions of the reaction, however, the slope changes are small and, over the higher ranges of substrate concentrations previously used when determination of initial velocities was easier, these would be negligible. For both directions of the reaction the substrates were varied in constant ratio and, as shown in Fig. II.8, the double reciprocal plots ob-



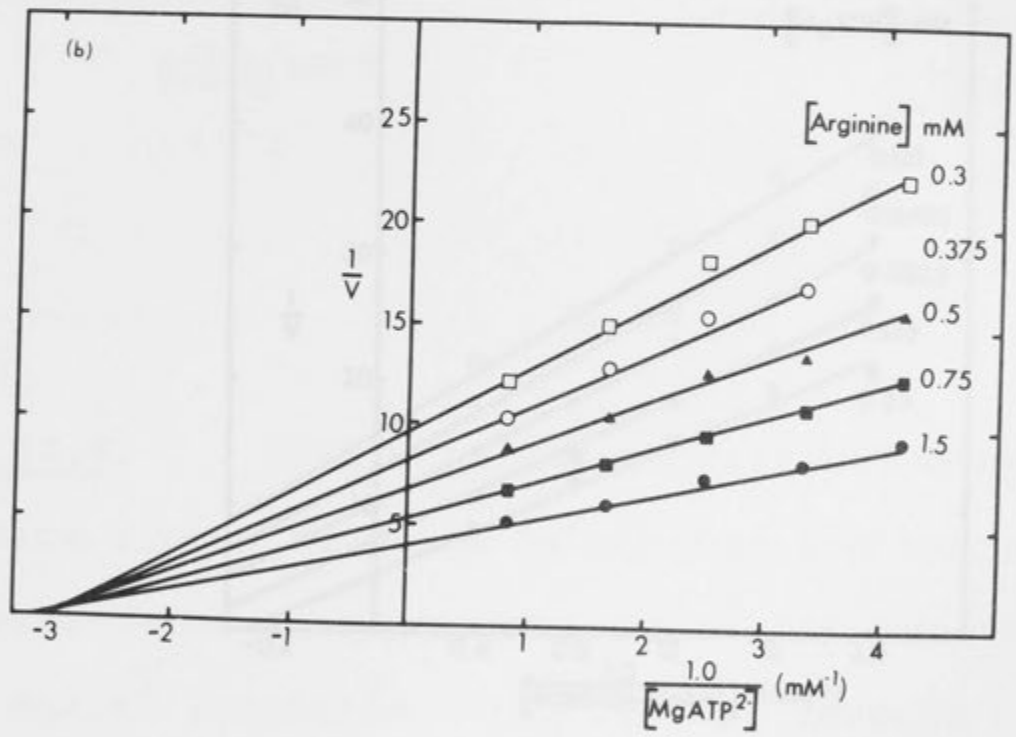
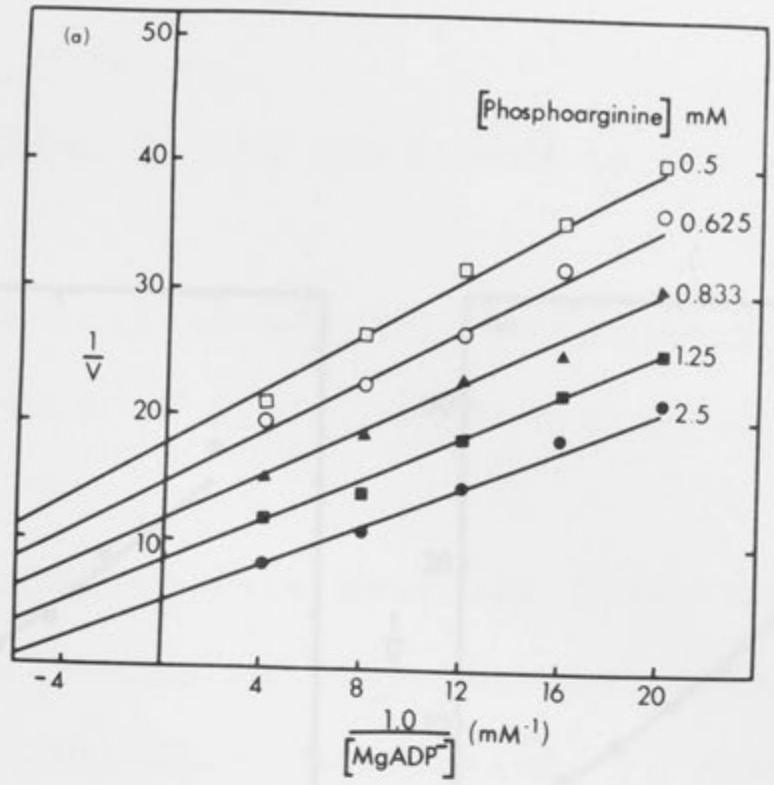


Fig. II.6. (a) Effect of $MgATP^{2-}$ concentration on the initial velocity of the forward reaction with arginine as the variable substrate. (b) Effect of arginine concentration on the initial velocity of the forward reaction with $MgATP^{2-}$ as the variable substrate. Velocities are expressed as μmoles per min per μg of arginine kinase.



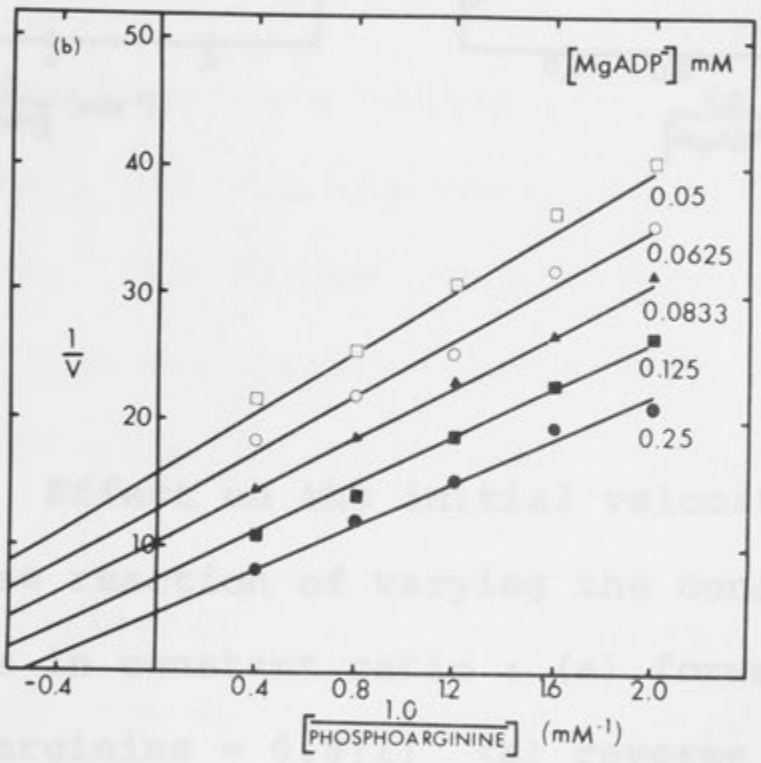


Fig. II.7. (a) Effect of phosphoarginine concentration on the initial velocity of the reverse reaction with $MgADP^-$ as the variable substrate. (b) Effect of $MgADP^-$ concentration on the initial velocity of the reverse reaction with phosphoarginine as the variable substrate. Velocities are expressed as μmoles per min per μg of arginine kinase.

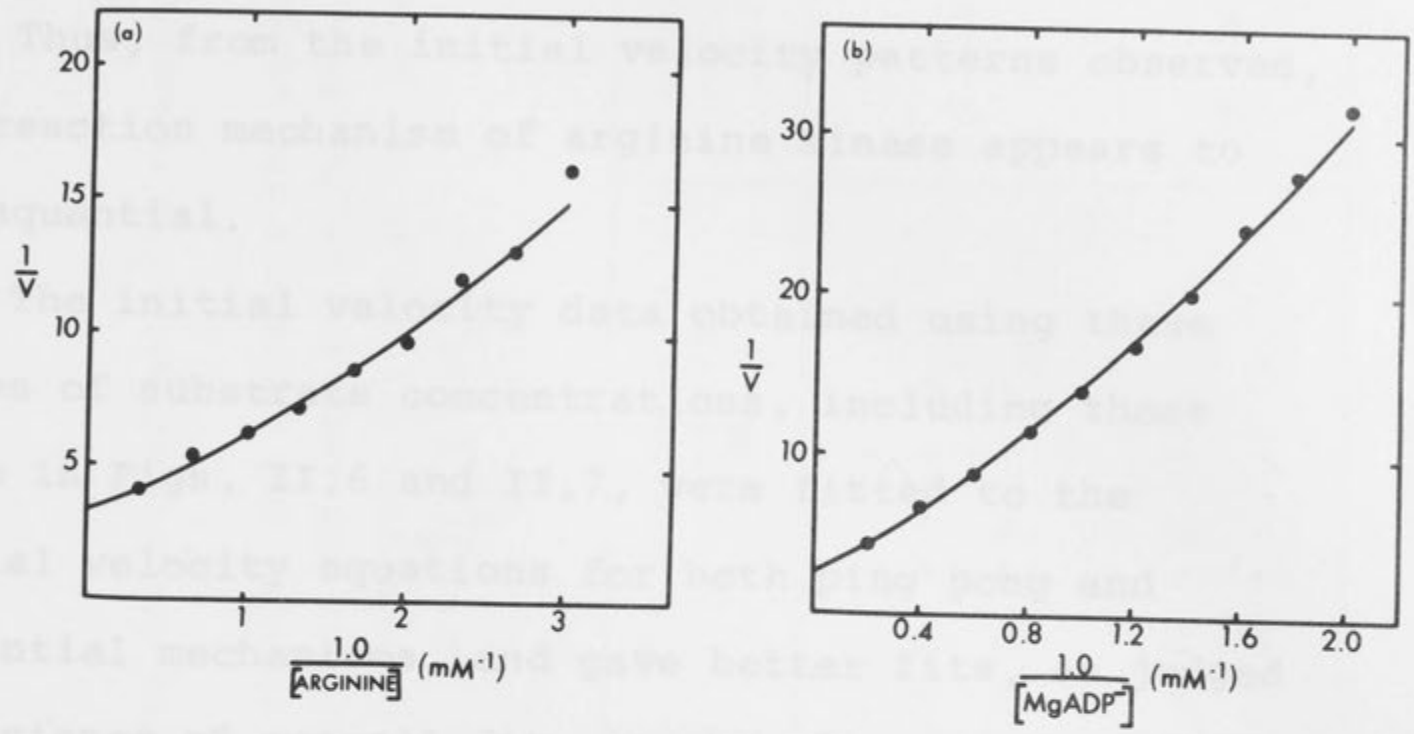


Fig. II.8. Effect on the initial velocity of the arginine kinase reaction of varying the concentrations of the substrates in constant ratio : (a) forward reaction with $MgATP^{2-}$:arginine = 0.8:1; (b) reverse reaction with phosphoarginine:$MgADP^{-}$ = 10:1. Velocities are expressed as μmoles per min per μg of arginine kinase.

tained were parabolic, giving good fits to the equation for a parabola.

Thus, from the initial velocity patterns observed, the reaction mechanism of arginine kinase appears to be sequential.

The initial velocity data obtained using these ranges of substrate concentrations, including those shown in Figs. II.6 and II.7, were fitted to the initial velocity equations for both ping pong and sequential mechanisms, and gave better fits, as judged by variance of overall fit, to the equation for a sequential mechanism. The values for the kinetic constants obtained from analysis of initial velocity data using the SEQUEN computer programme are given in Table II.2.

The initial velocity data previously obtained with higher substrate concentrations was also analysed, using both the PING PONG and SEQUEN computer programmes, and it was found that, when the data was fitted using the SEQUEN programme, the values for the dissociation constants (K_i) were meaningless. Also, it is interesting to note that the ratio of the maximum velocities of the forward and reverse reactions is less when the initial velocity data obtained with the lower ranges

Table II.2.

Values for the Michaelis and dissociation constants associated with the reaction of substrates with various forms of arginine kinase.

The values were determined from initial velocity studies and represent the weighted means of values obtained from three and six experiments for the reaction in the forward and reverse directions, respectively. Analyses were made by computer fitting of the data using the SEQUEN programme. It was assumed that $\frac{K_{ia}K_b}{K_a} = K_{ib}$ and $\frac{K_{ip}K_q}{K_p} = K_{iq}$.

Substrate	Range of substrate concentrations (mM)	Michaelis constant (mM)	Dissociation constant (mM)	Maximum velocity µmoles/min/µg of enzyme
Forward reaction:				
$MgATP^{2-}$	0.24 → 1.2	0.32 ± 0.03 (K_a)	0.34 ± 0.04 (K_{iq})	0.394 ± 0.015 (V_1)
Arginine	0.3 → 1.5	0.75 ± 0.06 (K_b)	0.81 ± 0.11 (K_{ib})	
Reverse reaction:				
Phosphoarginine	0.5 → 2.5	3.82 ± 0.39 (K_p)	0.26 ± 0.04 (K_{ip})	0.606 ± 0.046 (V_2)
$MgADP^-$	0.05 → 0.25	0.40 ± 0.04 (K_q)	0.024 ± 0.004 (K_{iq})	

of substrate concentrations are fitted using the SEQUEN programme, (0.65), than when the previous initial velocity data are fitted using the PING PONG programme (1.09).

Since the reaction mechanism of this arginine kinase has been shown to be sequential, isotope exchange studies at equilibrium were carried out to obtain more information about the mechanism.

Studies of isotope exchange at equilibrium

The equilibrium of the reaction was established enzymically, and the initial rates of the ATP-ADP and arginine-phosphoarginine exchanges were determined under three different sets of equilibrium conditions. These conditions are shown in Table II.4, with the concentrations of the free and Mg-complexed reactants. The results of isotope exchange studies at equilibrium, shown in Table II.3, indicate that there is a concomitant increase in both exchange rates with increased equilibrium concentrations of the reactants. In addition, it appears that the rates of the ADP-ATP and arginine-phosphoarginine exchanges are similar at the lowest equilibrium concentrations of the reactants. However, as these concentrations are increased, in Experiments II and III (Table II.3), the rate of the

Table II.3.

Rates of isotope exchange at equilibrium under three different sets of conditions.

Reaction mixtures (3.3 ml) contained the following components in the presence of 0.1 M triethanolamine buffer (pH 8.0) and 0.01 mM EDTA at 30° :- Experiment I : phosphoarginine, 0.5 mM; ADP, 0.5 mM; ATP, 0.11 mM; $MgCl_2$, 0.7 mM. Experiment II : phosphoarginine, 1.5 mM; ADP, 1.5 mM; ATP, 0.3 mM; $MgCl_2$, 2.1 mM. Experiment III : phosphoarginine, 5.0 mM; ADP, 5.0 mM; ATP, 1.0 mM; $MgCl_2$, 7.0 mM. Arginine kinase (0.5 µg per ml) was added to the reaction mixtures and, after establishment of equilibrium, which required 8, 12 and 25 min, with the components listed for Experiments I, II and III, respectively, two samples of 0.18 ml were withdrawn. To start the exchange reactions, 3 µl of [^{14}C]-L-arginine (0.08 µC; 0.44 mµmole) was added to one sample and 1 µl of [^{14}C]-ATP (0.08 µC; 2.8 mµmoles) to the other. Aliquots (40 µl) were taken at intervals between 2 and 20 min. The exchange reaction was stopped, and the initial rates were determined as described in the Methods section. The calculated exchange rates were determined using the equation :

$$v = \frac{V_1}{\frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1 + \frac{V_1}{V_2}\left\{\frac{K_p}{P} + \frac{K_q}{Q} + \frac{K_qA}{K_{Ia}Q} + \frac{K_pB}{K_{Ib}P} + 1\right\}}$$

Experiment	Determined exchange rate		Ratio of exchange rates	Calculated exchange rate
	Arginine-phosphoarginine	ATP-ADP		
	mµmoles/min/µg			mµmoles/min/µg
I	7.7 ± 0.2	7.0 ± 0.2	1.10 ± 0.04	18.6 ± 0.07
II	22.9 ± 1.1	14.9 ± 0.2	1.54 ± 0.08	41.7 ± 1.7
III	40.5 ± 3.4	20.7 ± 1.0	1.96 ± 0.19	80.5 ± 4.0

Table II.4.

Equilibrium concentrations of various species under conditions of the isotope exchange experiments and values for the apparent and true equilibrium constants of the reaction.

The experimental conditions were as given in Table II.3. The concentrations of the species were calculated as described under Methods, using the total concentration of magnesium and the equilibrium concentrations of the nucleotide and guanidino substrates. The apparent (K_{eq}') and true (K_{eq}) equilibrium constants were calculated from the relationships

$$K_{eq}' = \frac{[\text{phosphoarginine}][\text{ADP}]}{[\text{arginine}][\text{ATP}]} \quad \text{and} \quad K_{eq} = \frac{[\text{free phosphoarginine}][\text{MgADP}^-]}{[\text{arginine}][\text{MgATP}^{2-}]}$$

and are the mean values from either four or six experiments.

Experiment	Magnesium		ATP*		Arginine	ADP*		Phosphoarginine*		K_{eq}'	K_{eq} †
	Total	Free	Free	$MgATP^{2-}$		Free	$MgADP^-$	Free	Mg-complex		
	mM	mM	mM	mM	mM	mM	mM	mM	mM		
I	0.7	0.234	0.015	0.331	0.232	0.139	0.129	0.262	0.006	0.95 ± 0.07	0.44 ± 0.02
II	2.1	0.532	0.027	1.013	0.740	0.243	0.517	0.721	0.038	0.75 ± 0.06	0.50 ± 0.06
III	7.0	1.202	0.041	3.480	2.520	0.427	2.052	2.213	0.266	0.69 ± 0.03	0.52 ± 0.04

*The total equilibrium concentrations of these compounds are equal to the sum of the free and metal-complex species.

†Weighted mean value is 0.46 ± 0.02.

ATP-ADP exchange decreases relative to that of the arginine-phosphoarginine exchange. Since both exchanges are measured for the same direction of the reaction, these differences in rate cannot be due to failure to establish true equilibrium conditions (Morrison and White, 1967). The calculated rates of isotope exchange at equilibrium under these conditions are also shown in Table II.3. These were determined, assuming a rapid equilibrium, random mechanism with two dead-end complexes, by substituting values obtained from initial velocity studies (Table II.2) and product inhibition studies (Chapter III, Table III.4), into an equation analogous to equation 2 of Morrison and Cleland (1966), and given in the legend to Table II.3.

Equilibrium constant of reaction

In the experiments involving isotope exchange at equilibrium, the concentration of arginine at various times after the addition of enzyme was measured and, when this reached a constant value, it was assumed that equilibrium had been attained. Since the initial concentrations of phosphoarginine, ADP and ATP, and the equilibrium concentration of arginine were known, the equilibrium concentrations of all reactants could be determined. From these, the equilibrium concen-

trations of free phosphoarginine, $MgADP^-$ and $MgATP^{2-}$ were calculated. The results are given in Table II.4, and were used to determine values for both the apparent and true equilibrium constants for the reaction, which are also listed in this table. From these results it is clear that, whereas the apparent values vary with the equilibrium conditions, the true value remains virtually constant. The weighted mean value for K_{eq} of 0.46 $\pm$ 0.02 may be compared with 0.31 reported by Uhr *et al.* (1966).

DISCUSSION

The results obtained from initial velocity and qualitative partial isotope exchange studies with arginine kinase indicated that the reaction mechanism was ping pong. For this type of mechanism, the initial velocity equations for the forward and reverse reactions would be :-

$$v = \frac{V_1 AB}{K_b A + K_a B + AB} \qquad \text{(II.1.a)}$$

and

$$v = \frac{V_2 PQ}{K_q P + K_p Q + PQ} \qquad \text{(II.1.b)}$$

where v is the initial velocity and V_1 and V_2 are the maximum velocities in the forward and reverse directions, respectively. A, B, P and Q represent the concentrations of $MgATP^{2-}$, arginine, phosphoarginine and $MgADP^{-}$, and K_a, K_b, K_p and K_q are their respective Michaelis constants. Rearrangement of equation II.1.a, in double reciprocal form, gives :-

$$\frac{1}{v} = \frac{K_a}{V_1}\cdot\frac{1}{A} + \frac{1}{V_1}\left(\frac{K_b}{B} + 1\right) \qquad \text{(II.2)}$$

so that a plot of $1/v$ against $1/A$ yields a series of straight lines, whose slope is independent of the fixed concentration of B. The initial velocity equation

(II.1.a) may also be rearranged with the two substrates held in constant ratio (B = xA). In this case, the equation takes the form :-

$$\frac{1}{v} = \frac{1}{V_1}\left\{K_a + \frac{K_b}{x}\right\}\frac{1}{A} + \frac{1}{V_1} \qquad \text{(II.3)}$$

Thus, a double reciprocal plot of initial velocity against substrate concentration, when the substrates are held in constant ratio, is a straight line whose intercept on the vertical axis gives the value of the maximum velocity. The initial velocity patterns shown in Figs. II.1 and II.2 are, therefore, consistent with a ping pong mechanism. Further support for this mechanism was obtained when partial isotope exchanges were demonstrated between arginine and phosphoarginine, in the absence of nucleotides, and between ADP and ATP, in the absence of guanidino reactants. Since the rates of the partial exchanges were very low in relation to the rate of the overall reaction, further kinetic investigations were carried out, which showed the mechanism to be sequential. This result emphasises the need to exercise caution in the interpretation of kinetic data.

The reason for the confusion about the reaction mechanism can be deduced by examination of the initial

velocity equations, the kinetic constants for the reactants, and the substrate concentrations used in initial velocity studies.

For a sequential mechanism, the equations are :-

$$v = \frac{V_1 AB}{K_{ia}K_b + K_b A + K_a B + AB} \qquad (II.4.a)$$

and $$v = \frac{V_2 PQ}{K_{ip}K_q + K_q P + K_p Q + PQ} \qquad (II.4.b)$$

In double reciprocal form equation II.4.a becomes :-

$$\frac{1}{v} = \frac{1}{V_1}\left\{\frac{K_{ia}K_b}{B} + K_a\right\}\frac{1}{A} + \frac{1}{V_1}\left\{\frac{K_b}{B} + 1\right\} \qquad (II.5)$$

If the constant term, $K_{ia}K_b$, becomes so small as to be negligible, equation II.4.a reduces to that for a ping pong mechanism (equation II.1.a) and parallel double reciprocal plots are obtained. From the kinetic data, presented in Table II.2, it can be seen that the range of substrate concentrations used in the first set of initial velocity studies ($MgATP^{2-}$ (A), 0.48 to 2.4 mM; arginine (B), 0.6 to 3.0 mM) was high relative to both the K_i and the K_m values for both substrates. For this reason, the value of the constant term, $K_{ia}K_b$ or $K_{ib}K_a$, became insignificant in relation to the

terms involving A and B, so that the equation approximated to that for a ping pong mechanism. In the reverse direction, on the other hand, the substrates phosphoarginine (P) and $MgADP^-$ (Q) were varied over a range appropriate to their K_m values, so that intersecting initial velocity patterns would have been expected. However, because the dissociation constants for both these substrates (K_{ip} and K_{iq}) are very small relative to the Michaelis constants and the substrate concentrations used, such patterns were not obtained. That is, the constant term, $K_{ip}K_q$ or $K_{iq}K_p$, again becomes negligible with respect to the other terms in the equation, so that the equation has the form of that for a ping pong mechanism.

The fact that the mechanism is not ping pong was detected when AMP was used as a dead-end inhibitor, because the constant term in equation II.4.b became significant when multiplied by a factor containing the concentration of the inhibitor. When both substrates of the reverse reaction are varied in constant ratio (P = xQ), the double reciprocal plot must be parabolic, since the equation takes the form :-

$$\frac{1}{v} = \frac{1}{V_2}\left\{\frac{K_{ip}K_q}{x}\right\}\frac{1}{Q^2} + \frac{1}{V_2}\left\{\frac{K_p}{x} + K_q\right\}\frac{1}{Q} + \frac{1}{V_2} \qquad \text{(II.6.b)}$$

If $K_{ip}K_q$ is so small as to be negligible, a straight line is obtained but, if the inhibitor combines with the free enzyme to form a dead-end complex, the Q^2 term becomes significant and a parabola is obtained. Under these conditions, equation II.6.b becomes :-

$$\frac{1}{v} = \frac{1}{V_2}\left\{\frac{K_{ip}K_q}{x}\left[1 + \frac{I}{K_I}\right]\right\}\frac{1}{Q^2} + \frac{1}{V_2}\left\{\frac{K_p}{x} + K_q\right\}\frac{1}{Q} + \frac{1}{V_2} \qquad \text{(II.7)}$$

where I is the inhibitor and K_I is the dissociation constant for the interaction of the inhibitor with the free enzyme.

Thus it may be possible, using a dead-end inhibitor and varying the substrates in constant ratio, to detect non-linearity in double reciprocal plots when an enzyme has a sequential mechanism but parallel initial velocity patterns have been obtained.

Parallel initial velocity patterns, arising from the use of substrate concentrations which are high relative to the Michaelis constants of the substrates, have recently been reported for hypoxanthine-guanine phosphoribosyltransferase by Henderson et al. (1968). These authors showed by isotope exchange and product inhibition studies that, although double reciprocal

plots of initial velocity against substrate concentration yield families of parallel lines, the reaction mechanism is, in fact, ordered sequential. Packman and Jakoby (1965) reported that crude preparations of quinolate phosphoribosyltransferase give parallel initial velocity patterns. However, later investigations, using a more highly purified enzyme preparation and lower substrate concentrations, showed that the lines in a double reciprocal plot intersect to the left of the vertical axis (Packman and Jakoby, 1967). Henderson et al. (1968) also indicate that, although parallel initial velocity patterns have been reported for adenine phosphoribosyltransferase by Hori and Henderson (1966), it is probable that this enzyme also has a sequential reaction mechanism, involving the formation of a ternary complex of both substrates with the enzyme.

Toews (1966) was the first to realise that this type of situation might arise. He suggested that, although Hanson and Fromm (1965) had varied the ATP and glucose concentrations over a wide range, these concentrations were high relative to the K_m values, and intersecting lines in double reciprocal plots of initial velocity against substrate concentration might

have been observed had the substrate concentrations been varied over ranges near the K_m values. It now seems that parallel initial velocity patterns alone are not sufficient to identify a ping pong mechanism, and that further quantitative studies are required to confirm such a conclusion.

From initial velocity studies of the reaction of arginine kinase from H. vulgaris, Virden et al. (1965) concluded that the mechanism is sequential, since converging lines were obtained. Lacombe et al. (1969), investigating an arginine kinase with a molecular weight of approximately 80,000, isolated from the siponcle, Sipunculus nudus, report parallel initial velocity patterns and suggest that the reaction has a ping pong mechanism. When considering the conclusions drawn from this study it should be noted that the authors used relatively high substrate concentrations (arginine, 1 to 10 mM; $MgATP^{2-}$, 0.625 to 2.5 mM) (Table II.5), and initial velocity studies were carried out for one direction of the reaction only. However, since the values of the kinetic constants for these arginine kinases have not been determined, it is not possible to compare the ranges of substrate concentrations used in initial velocity studies with these constants.

Table II.5.

The initial velocity patterns obtained, and the ranges of substrate concentrations used in their determination, for the forward reaction of arginine kinases from a number of sources.

Source of arginine kinase	Initial velocity pattern	$MgATP^{2-}$ (mM)	Arginine (mM)	References
H. vulgaris	Intersecting	0.67 → 5.0	0.4 → 5.0	Virden et al. (1965)
H. vulgaris	Parallel	1.25 → 5.0	1.66 → 10.0	Lacombe et al. (1969)
S. nudus	Parallel	0.625 → 2.5	1.0 → 10.0	Lacombe et al. (1969)
J. verreauxi	Parallel	0.48 → 2.4	0.6 → 3.0	Uhr et al. (1966)
P. longipes	Parallel	0.48 → 2.4	0.6 → 3.0	Smith and Morrison (1969)
	Intersecting	0.24 → 1.2	0.3 → 1.5	Smith and Morrison (1969)

Initial velocity and qualitative partial isotope exchange studies with arginine kinase from J. verreauxi have been reported by Uhr et al. (1966), and the results appear similar to those obtained in this investigation (Table II.5).

The initial velocity studies over the lower range of substrate concentrations, reported in this chapter, indicate that the reaction mechanism of arginine kinase from P. longipes is sequential. The greatly increased rate of isotope exchange in the presence of all substrates is also in accord with a sequential mechanism. Since the rates of isotope exchange for the two pairs of substrates are very similar (Table II.3), it seems probable that the system is in rapid equilibrium, with the interconversion of the ternary complex the slowest step in the reaction sequence. However, if the system is truly rapid equilibrium, the two exchange rates should be equal, irrespective of the equilibrium concentrations of the reactants. In fact, as the concentrations of the reactants are increased, the rate of the arginine-phosphoarginine exchange increases at a greater rate than does the ATP-ADP exchange. It is possible that, in Experiments II and III of Table II.3, the equilibrium concentrations of $MgADP^-$ (Table II.4)

were sufficiently high to cause the formation of a complex similar to that formed when this nucleotide causes substrate inhibition. The interaction of $MgADP^-$ with the central enzyme-phosphoarginine-MgADP complex might give rise to such a complex, from which phosphoarginine and $MgADP^-$ are released at different rates. In addition, comparison of the experimental and calculated exchange rates shows the experimental values to be less than those calculated, using kinetically determined constants. These discrepancies could be accounted for, in part, by the fact that the equation used to calculate the exchange rates did not make allowance for the probable inhibitory effects of the ionic species, Cl^-, free Mg^{2+} and free ADP^{3-}, which are present in greater concentrations at the higher reactant concentrations (Morrison and Cleland, 1966). Since the kinetics of the inhibition of the reaction by these ionic species have not been investigated, values for the inhibition constants are not available to test this hypothesis.

Thus, although initial velocity and isotope exchange studies have indicated that the reaction mechanism is sequential and probably rapid equilibrium, no more definite conclusion can be drawn from these results alone.

However, from these studies and from product inhibition studies (Chapter III), it appears that the reaction mechanism is rapid equilibrium random, with two dead-end complexes. On the basis of this assumption, physical significance can be attributed to the kinetic constants, and the relationships, $K_{ia}K_b = K_{ib}K_a$ and $K_{ip}K_q = K_{iq}K_p$ are valid. K_{ia}, K_{ib}, K_{ip} and K_{iq} are dissociation constants for the reaction of A, B, P and Q with the free enzyme, and the Michaelis constants, K_a, K_b, K_p and K_q are the dissociation constants for the reaction of A, B, P and Q with EB, EA, EQ and EP, respectively, where E represents the enzyme.

It has been pointed out by Morrison and James (1965) that, if the reaction mechanism is rapid equilibrium random and the values of the kinetic constants are determined by fitting the initial velocity data to equations II.4, a and b, there is, in effect, only one Haldane relationship, which can be expressed as :-

$$K_{eq} = \frac{V_1 K_{ip} K_q}{V_2 K_{ia} K_b} \qquad (II.8)$$

When the values for $\frac{K_{ia}K_b}{V_1}$ and $\frac{K_{ip}K_q}{V_2}$, from the output of the computer programme which fits the initial velocity data to equations II.4, a and b, are substit-

uted into this equation, the value of K_{eq} is found to be 0.27 $\pm$ 0.04. This result is the weighted mean of values from four experiments in each direction. The value is in poor agreement with that obtained directly (0.46 $\pm$ 0.02, Table II.4), but is very close to that determined directly by Uhr *et al*. (1966).

SUMMARY

1. Initial velocity and isotope exchange studies were carried out on arginine kinase from P. longipes.

2. For the range of substrate concentrations at first used, initial velocity studies indicated that the enzyme had a ping pong reaction mechanism. Also, partial isotope exchanges were demonstrated.

3. At lower substrate concentrations, initial velocity studies indicated that the reaction mechanism is, in fact, sequential.

4. Isotope exchange studies at equilibrium yielded results consistent with a sequential reaction mechanism, close to, if not truly, rapid equilibrium.

CHAPTER III

Product Inhibition Studies of the Arginine Kinase Reaction

INTRODUCTION

The results of the initial velocity studies presented in Chapter II indicate that the reaction mechanism of the arginine kinase from the tail muscle of P. longipes is sequential. In addition, from the results of isotope exchange studies at equilibrium, it seems that the mechanism is close to rapid equilibrium, but no further information about the reaction mechanism can be obtained from initial velocity and these isotope exchange studies. However, a kinetic study of the effects of products of the reaction as inhibitors can usually give additional information about the mechanism of a sequential reaction.

The fact that the products of an enzymic reaction can function as inhibitors of that reaction was realised at an early stage in the development of enzyme kinetics (Henri, 1903; Michaelis and Menten, 1913). However, the potential of the phenomenon was not exploited for the elucidation of the reaction mechanisms of bireactant enzymes until Alberty (1958) showed that such investigations could be used to differentiate between various types of sequential mechanisms. Walter and Frieden (1963) have summarised developments in the analysis of product inhibition and emphasise

the fact that a product of a reaction is not necessarily competitive with respect to a substrate, even though both reactants may have similar chemical structures and bind to the same site on the enzyme.

Fromm and Nelson (1962) have discussed the effects of products as inhibitors, when the inhibition is due to the formation of a dead-end complex. Such a complex can break down only to yield the reactants from which it is formed, and occurs when the product combines with an enzyme form other than the one with which it reacts as a substrate in the reverse direction. Mixed dead-end and product inhibition by the one reactant results in non-linear double reciprocal plots.

The product inhibition patterns can be predicted quickly from inspection of a proposed reaction mechanism, when no alternative reaction sequences can occur (Cleland, 1963c). Alternatively, as pointed out by Cleland (1963a, 1963b), when the complete rate equation for a mechanism has been derived it can be altered by setting one or more of the product concentrations equal to zero. Rearrangement of the equation so obtained into double reciprocal form, with each substrate in turn as the variable one, then allows one to predict the product inhibition patterns. Also, the

meanings of the slope and/or intercept variations are apparent from the equations obtained. Cleland (1963a) has tabulated the product inhibition patterns expected for a number of bireactant mechanisms.

In this study, both products of the reverse reaction, arginine and $MgATP^{2-}$, and one product of the forward reaction, phosphoarginine, were used as inhibitors. $MgADP^{-}$ was not used as a product inhibitor of the forward reaction because of the difficulties involved in the accurate assay of phosphoarginine, the other product of the reaction (Uhr, 1966).

EXPERIMENTAL PROCEDURE

Materials

Materials were as described in Chapter II. In addition, NADP in the form of the sodium salt, hexokinase and glucose-6-phosphate dehydrogenase were obtained from Calbiochem.

Methods

Assays for arginine and ADP were carried out as described previously (Chapter I, p. 37, 38). ATP was estimated by measurement of the formation of NADPH from NADP in the presence of glucose, by a coupled enzyme system comprising hexokinase and glucose-6-phosphate dehydrogenase. The method is described in detail by Morrison and James (1965). However, all ATP assays with this system were commenced within 15 min of stopping the arginine kinase reaction, since anomalous results were obtained when the tubes were allowed to stand for longer periods. All reactions were run for at least two time periods, to ensure that initial rates were being measured.

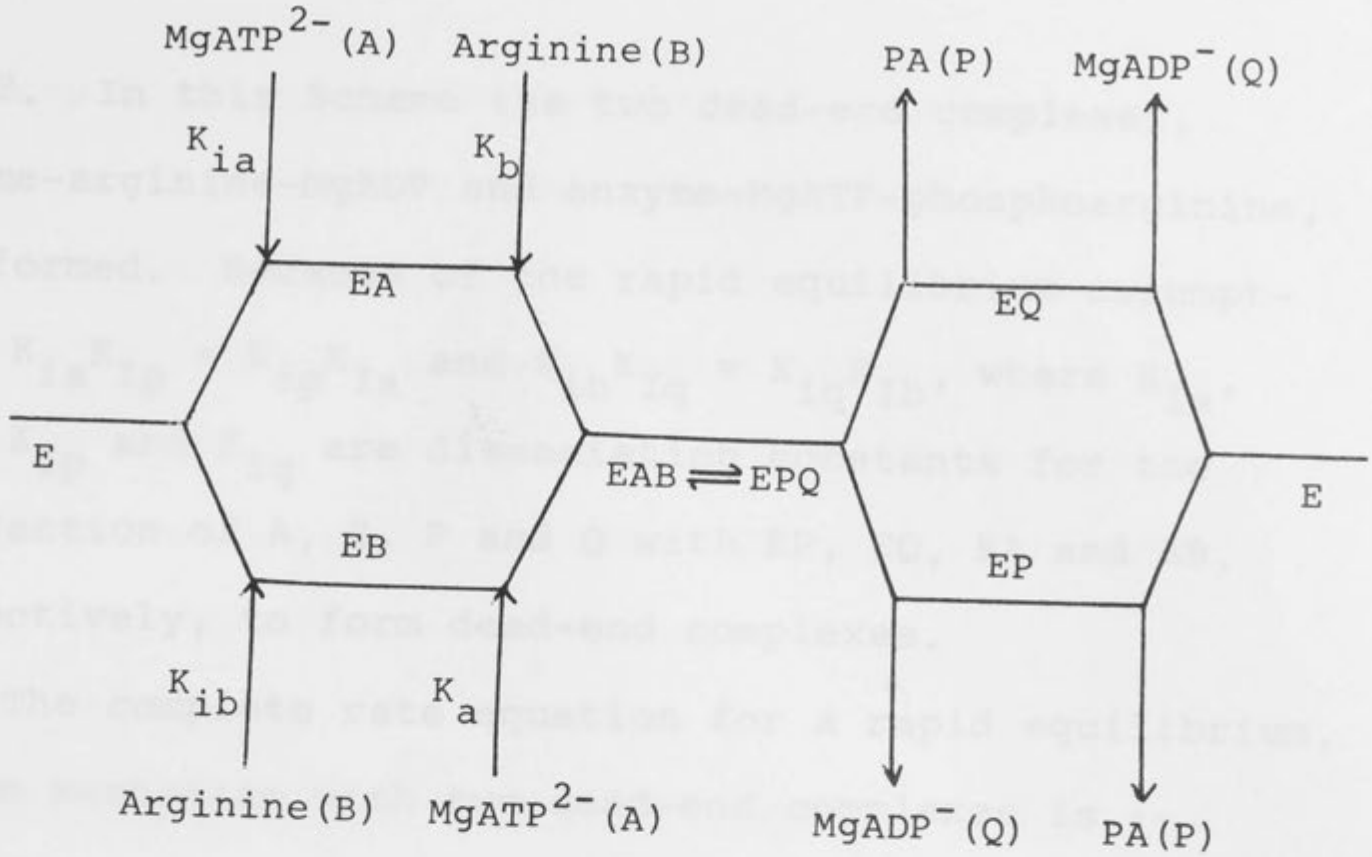
Analysis of kinetic data

The kinetic data was analysed as described in Chapter II, using the computer programmes COMP and NONCOMP (Preface, equations 6 and 7) in conjunction with the IBM 360 computer. The values of the kinetic constants and their standard errors were determined as described in Chapter II (p. 60).

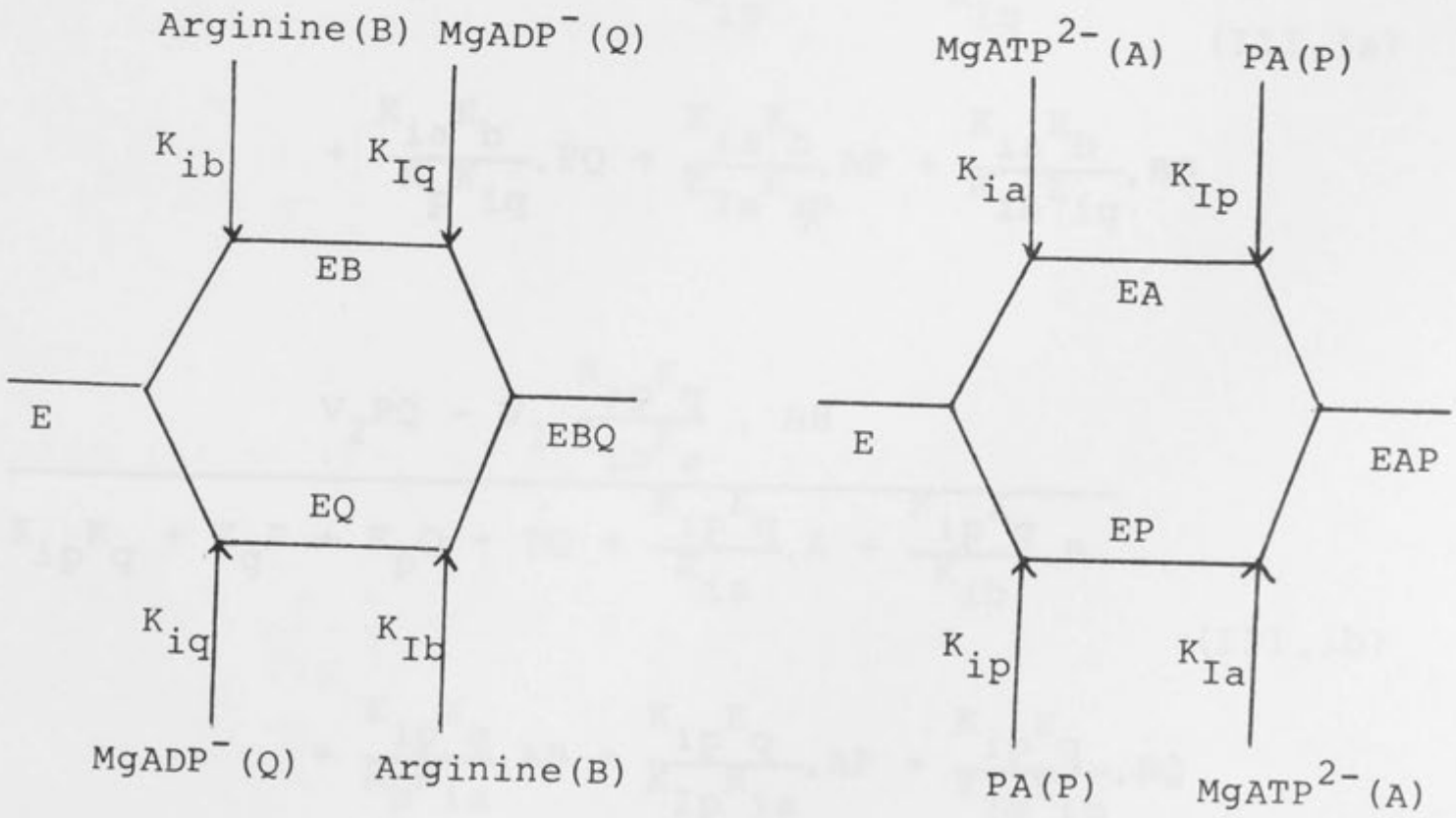
THEORY

In order to facilitate the presentation of the results of product inhibition studies with arginine kinase, it is useful to assume the reaction mechanism of the enzyme and justify the assumption later, on the basis of the results obtained. As shown by initial velocity and isotope exchange studies (Chapter II), the mechanism is sequential and close to rapid equilibrium. It is now assumed that the mechanism is rapid equilibrium, random. This mechanism is illustrated in Scheme III.1 on the following page, where phospho-arginine is represented by PA. For this type of mechanism, it is assumed that each catalytic centre on the enzyme comprises two distinct binding sites for the nucleotide and guanidino substrates. The inter-conversion of the ternary complexes is the slowest step in the reaction sequence, so that all other inter-actions are in rapid equilibrium.

From this rapid equilibrium assumption, it follows that $K_{ia}K_b = K_{ib}K_a$ and $K_{ip}K_q = K_{iq}K_p$, where K_{ia}, K_{ib}, K_{ip} and K_{iq} are the dissociation constants for the interaction of A, B, P and Q with EB, EA, EQ and EP, respectively. It is also to be assumed that two dead-end complexes can be formed. These are shown in Scheme



Scheme III.1



Scheme III.2

III.2. In this Scheme the two dead-end complexes, enzyme-arginine-MgADP and enzyme-MgATP-phosphoarginine, are formed. Because of the rapid equilibrium assumption, $K_{ia}K_{Ip} = K_{ip}K_{Ia}$ and $K_{ib}K_{Iq} = K_{iq}K_{Ib}$, where K_{Ia}, K_{Ib}, K_{Ip} and K_{Iq} are dissociation constants for the interaction of A, B, P and Q with EP, EQ, EA and EB, respectively, to form dead-end complexes.

The complete rate equation for a rapid equilibrium, random mechanism with two dead-end complexes is :-

$$v = \frac{V_1AB - V_2\frac{K_{ia}K_b}{K_{iq}K_p}.PQ}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_b}{K_{ip}}P + \frac{K_{ia}K_b}{K_{iq}}Q + \frac{K_{ia}K_b}{K_pK_{iq}}.PQ + \frac{K_{ia}K_b}{K_{Ia}K_{ip}}.AP + \frac{K_{ia}K_b}{K_{Ib}K_{iq}}.BQ} \qquad \text{(III.1a)}$$

$$v = \frac{V_2PQ - V_1\frac{K_{ip}K_q}{K_{ib}K_a}.AB}{K_{ip}K_q + K_qP + K_pQ + PQ + \frac{K_{ip}K_q}{K_{ia}}.A + \frac{K_{ip}K_q}{K_{ib}}.B + \frac{K_{ip}K_q}{K_bK_{ia}}.AB + \frac{K_{ip}K_q}{K_{Ip}K_{ia}}.AP + \frac{K_{ip}K_q}{K_{Iq}K_{ib}}.BQ} \qquad \text{(III.1b)}$$

From equation III.1a it is clear that, when no products are present, i.e. P = Q = 0, the initial

velocity equation is that given for a sequential mechanism in Chapter II (equation II.4.a). The complete rate equation (equations III.1a or III.1b) can also be modified by setting the concentration of one of the products equal to zero, so that the product inhibition patterns expected when the other product is present can be determined.

Forward reaction

Phosphoarginine as the product inhibitor, (Q = 0)

$$v = \frac{V_1 AB}{K_{ia}K_b + K_b A + K_a B + AB + \frac{K_{ia}K_b P}{K_{ip}} + \frac{K_{ia}K_b AP}{K_{ia}K_{Ip}}} \qquad (III.2)$$

$MgATP^{2-}$ as the variable substrate :-

$$\frac{1}{v} = \frac{K_a}{V_1}\left\{1 + \frac{K_b}{B}\left[1 + \frac{P}{K_{ip}}\right]\right\}\frac{1}{A} + \frac{1}{V_1}\left\{1 + \frac{K_b}{B}\left[1 + \frac{P}{K_{Ip}}\right]\right\} \qquad (III.3)$$

Arginine (B) as the variable substrate :-

$$\frac{1}{v} = \frac{K_b}{V_1}\left\{\left[1 + \frac{P}{K_{Ip}}\right] + \frac{K_{ia}}{A}\left[1 + \frac{P}{K_{ip}}\right]\right\}\frac{1}{B} + \frac{1}{V_1}\left\{1 + \frac{K_a}{A}\right\} \qquad (III.4)$$

Reverse reaction

$MgATP^{2-}$ (A) as product inhibitor, (B = 0)

$$v = \frac{V_2 PQ}{K_{ip}K_q + K_pQ + K_qP + PQ + \frac{K_{ip}K_qA}{K_{ia}} + \frac{K_{ip}K_qAP}{K_{Ia}K_{ip}}} \qquad (III.5)$$

Phosphoarginine (P) as the variable substrate :-

$$\frac{1}{v} = \frac{K_p}{V_2}\left\{1 + \frac{K_{iq}}{Q}\left[1 + \frac{A}{K_{ia}}\right]\right\}\frac{1}{P} + \frac{1}{V_2}\left\{1 + \frac{K_q}{Q}\left[1 + \frac{A}{K_{Ia}}\right]\right\} \qquad (III.6)$$

$MgADP^-$ (Q) as the variable substrate :-

$$\frac{1}{v} = \frac{K_q}{V_2}\left\{\left[1 + \frac{A}{K_{Ia}}\right] + \frac{K_{ip}}{P}\left[1 + \frac{A}{K_{ia}}\right]\right\}\frac{1}{Q} + \frac{1}{V_2}\left\{1 + \frac{K_p}{P}\right\} \qquad (III.7)$$

Arginine (B) as the product inhibitor, (A = 0)

$$v = \frac{V_2 PQ}{K_{ip}K_q + K_pQ + K_qP + PQ + \frac{K_{iq}K_pB}{K_{ib}} + \frac{K_{iq}K_pBQ}{K_{iq}K_{Ib}}} \qquad (III.8)$$

Phosphoarginine (P) as the variable substrate :-

$$\frac{1}{v} = \frac{K_p}{V_2}\left\{\left[1 + \frac{B}{K_{Ib}}\right] + \frac{K_{iq}}{Q}\left[1 + \frac{B}{K_{ib}}\right]\right\}\frac{1}{P} + \frac{1}{V_2}\left\{1 + \frac{K_q}{Q}\right\} \qquad \text{(III.9)}$$

$MgADP^-$ (Q) as the variable substrate :-

$$\frac{1}{v} = \frac{K_q}{V_2}\left\{1 + \frac{K_{ip}}{P}\left[1 + \frac{B}{K_{ib}}\right]\right\}\frac{1}{Q} + \frac{1}{V_2}\left\{1 + \frac{K_p}{P}\left[1 + \frac{B}{K_{Ib}}\right]\right\} \qquad \text{(III.10)}$$

The types of inhibition predicted by these equations are set out in Table III.1.

Table III.1.

Product inhibition patterns predicted on the assumption of a rapid equilibrium, random mechanism for the arginine kinase reaction.

Forward reaction

Product Inhibitor	Variable Substrate	Inhibition Pattern	Equation
Phospho-arginine	$MgATP^{2-}$	Non-competitive	III.3
	Arginine	Competitive	III.4

Reverse reaction

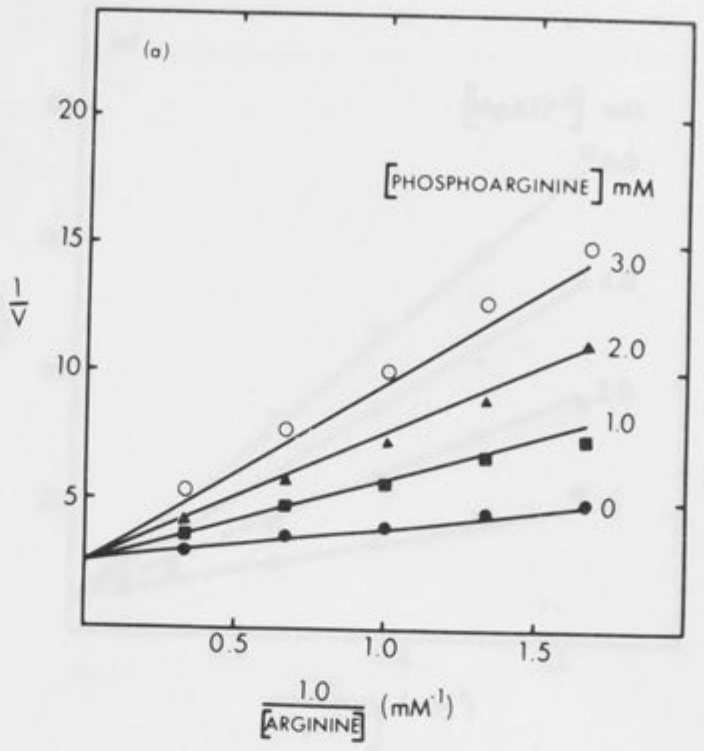
Product Inhibitor	Variable Substrate	Inhibition Pattern	Equation
$MgATP^{2-}$	Phosphoarginine	Non-competitive	III.6
	$MgADP^{-}$	Competitive	III.7
Arginine	Phosphoarginine	Competitive	III.9
	$MgADP^{-}$	Non-competitive	III.10

RESULTS

Product inhibition studies were carried out with the three reactants, $MgATP^{2-}$, arginine and phosphoarginine as inhibitors. The results of inhibition of the forward reaction by phosphoarginine, with arginine and $MgATP^{2-}$ as the variable substrates, are shown in Fig. III.1. The patterns obtained with $MgATP^{2-}$ and arginine as inhibitors of the reverse reaction are shown in Figs. III.2 and III.3, respectively. In all cases the inhibition patterns are those expected for a rapid equilibrium, random mechanism with two dead-end complexes. Since the inhibition patterns are symmetrical, it seemed likely that the information gained from product inhibition by $MgADP^{-}$ would not be essential in determining the reaction mechanism of the enzyme.

The lines in all the non-competitive inhibition patterns intersect close to the vertical axis, and these data were, therefore, analysed by computer using both the COMP and NONCOMP programmes. The results showed better fits to the equation for non-competitive inhibition in all cases.

Assuming, then, that the mechanism is rapid equilibrium, random with two dead-end complexes, the



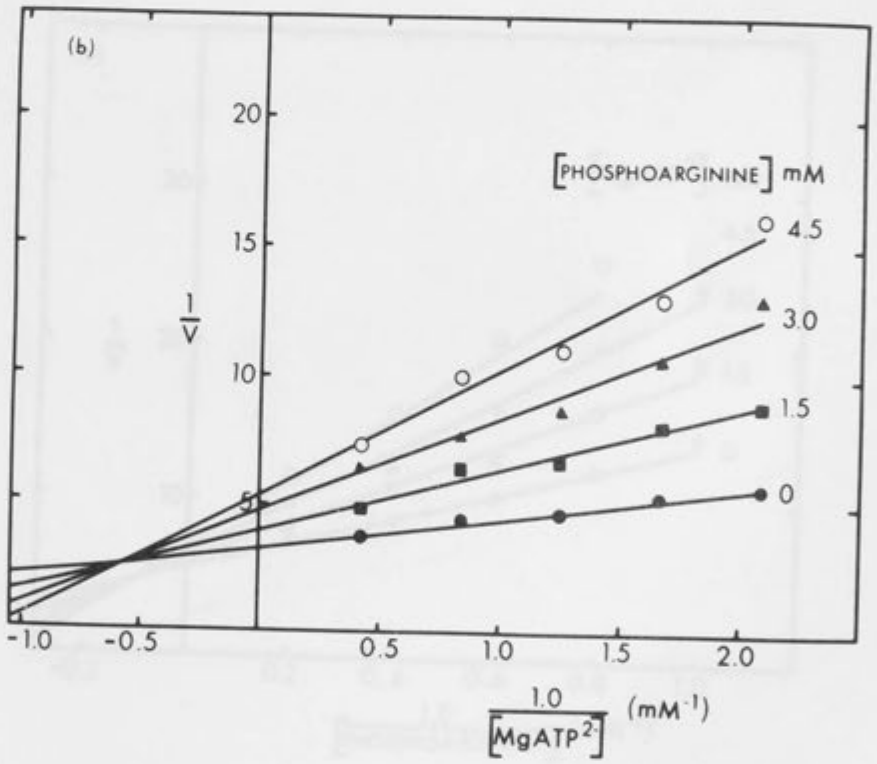
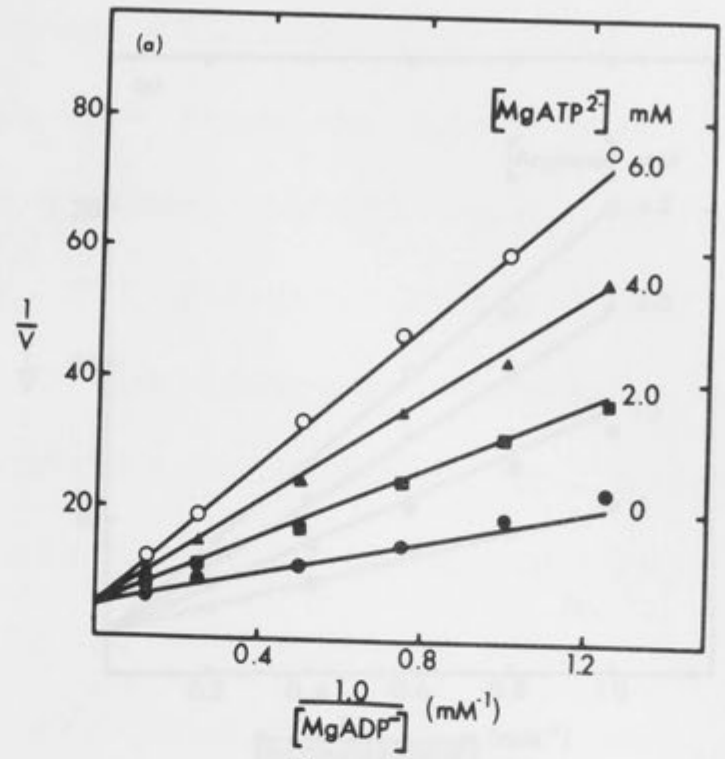


Fig. III.1. Product inhibition of the forward reaction by phosphoarginine. (a) Arginine as the variable substrate and $MgATP^{2-}$ held constant at 1.2 mM. (b) $MgATP^{2-}$ as the variable substrate and arginine held constant at 1.5 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.



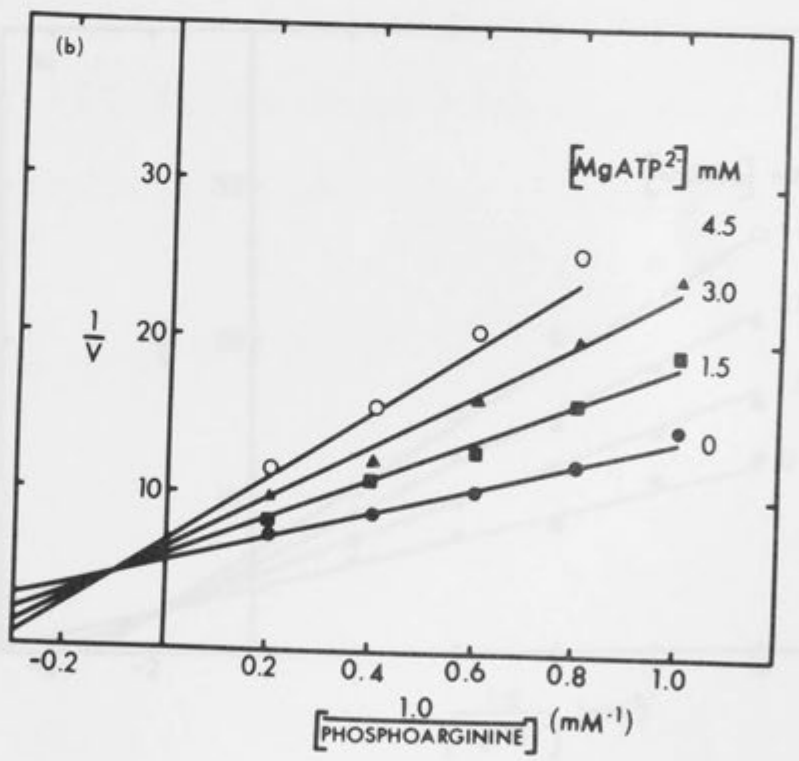
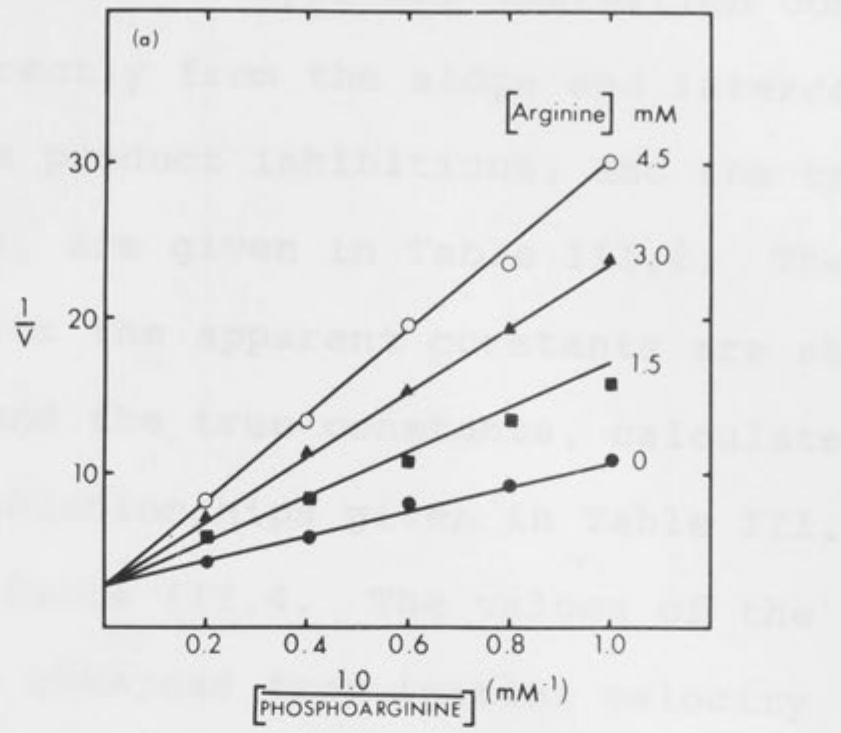


Fig. III.2. Product inhibition of the reverse reaction by $MgATP^{2-}$. (a) $MgADP^{-}$ as the variable substrate and phosphoarginine held constant at 2.5 mM. (b) Phosphoarginine as the variable substrate and $MgADP^{-}$ held constant at 2.5 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.



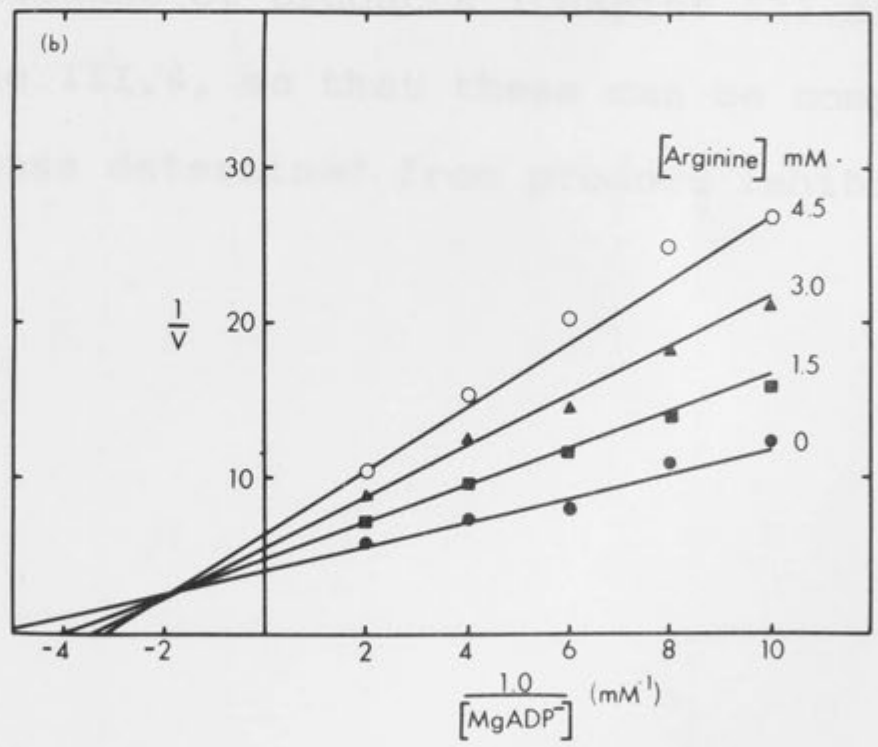


Fig. III.3. Product inhibition of the reverse reaction by arginine. (a) Phosphoarginine as the variable substrate and $MgADP^-$ held constant at 0.25 mM. (b) $MgADP^-$ as the variable substrate and phosphoarginine held constant at 2.5 mM. Velocities are expressed as µmoles per min per µg of arginine kinase.

relationships between the apparent inhibition constants, as determined directly from the slope and intercept variations in the product inhibitions, and the true kinetic constants, are given in Table III.2. The values obtained for the apparent constants are shown in Table III.3, and the true constants, calculated from these using the relationships given in Table III.2, are presented in Table III.4. The values of the kinetic constants obtained from initial velocity studies in the absence of products (Chapter II) are included in Table III.4, so that these can be compared directly with those determined from product inhibition studies.

Table III.2.

Relationships between the apparent inhibition constants, as determined from competitive and non-competitive inhibitions by products, and the true constants for a rapid equilibrium, random mechanism with two dead-end complexes.

Product Inhibitor	Variable Substrate	Type of Inhibition	Relationship between kinetic constants from		
			Slope replot (K_{is})	Intercept replot (K_{ii})	Crossover point
P	A	N.C.*	$K_{ip} = K_{is}\left(\frac{K_{ib}}{K_{ib} + B}\right)$	$K_{Ip} = K_{ii}\left(\frac{K_b}{K_b + B}\right)$	K_{Ia}
	B	C	$K_{Ip} = K_{is}\left(\frac{K_{Ia} + A}{K_{ia} + A}\right)$		
A	P	N.C.	$K_{ia} = K_{is}\left(\frac{K_{iq}}{K_{iq} + Q}\right)$	$K_{Ia} = K_{ii}\left(\frac{K_q}{K_q + Q}\right)$	K_{Ip}
	Q	C	$K_{Ia} = K_{is}\left(\frac{K_{Ip} + P}{K_{ip} + P}\right)$		
B	P	C	$K_{Ib} = K_{is}\left(\frac{K_{Iq} + Q}{K_{iq} + Q}\right)$		
	Q	N.C.	$K_{ib} = K_{is}\left(\frac{K_{ip}}{K_{ip} + P}\right)$	$K_{Ib} = K_{ii}\left(\frac{K_p}{K_p + P}\right)$	K_{Iq}

*N.C. = non-competitive; C = competitive

Table III.3.

Values of apparent inhibition constants determined from competitive and non-competitive product inhibitions. The experimental data were fitted by the COMP and NONCOMP computer programmes and the constants are expressed as mM. Each value for K_{ii} and K_{is} is the weighted mean of values from at least two experiments.

Product Inhibitor	Variable Substrate	Type of Inhibition	K_{is}	Concentration of Fixed Substrate	K_{ii}	Concentration of Fixed Substrate
P	A	N.C.*	1.50 ± 0.17	B = 1.5 mM	5.70 ± 0.67	B = 1.5 mM
	B	C	0.81 ± 0.04 0.73 ± 0.04	A = 2.4 mM A = 1.2 mM		
A	P	N.C.	2.47 ± 0.16	Q = 0.25 mM	13.14 ± 2.03	Q = 0.25 mM
	Q	C	2.11 ± 0.07 1.28 ± 0.06	P = 2.5 mM P = 1.25 mM		
B	P	C	1.72 ± 0.27	Q = 0.25 mM		
	Q	N.C.	2.57 ± 0.31	P = 2.5 mM	7.12 ± 1.41	P = 2.5 mM

*N.C. = non-competitive; C = competitive

Table III.4.

Summary of values of Michaelis and dissociation constants associated with the reaction of substrates with various forms of arginine kinase.

The values given in the first two columns were determined from initial velocity studies in the absence of products and are given in Table II.2. The true product inhibition constants were obtained from the vertical co-ordinate of the crossover point for the non-competitive inhibitions, and by calculation using the relationships given in Table III.2. and values given in Table III.3. All values are expressed in mM.

Substrate	Michaelis Constant (K_m)	Dissociation Constant (K_i)		Dissociation Constant (K_I)		
		Initial velocity data	Product inhibition data (non-competitive)	Product inhibition data		Crossover point
				Competitive	Non-competitive	
$MgATP^{2-}$ (A)	0.32 ± 0.03	0.34 ± 0.04	0.22 ± 0.04	2.43 ± 0.12†		1.43 ± 0.26
Arginine (B)	0.75 ± 0.06	0.81 ± 0.11	0.24 ± 0.05	3.45 ± 0.48	4.30 ± 0.97	
Phospho-arginine (P)	3.82 ± 0.39	0.26 ± 0.04	0.32 ± 0.06	1.46 ± 0.11†	1.14 ± 0.16	
$MgADP^{-}$ (Q)	0.40 ± 0.04	0.024 ± 0.004				0.55 ± 0.14

†Weighted mean of two values obtained at different concentrations of the fixed substrate.

DISCUSSION

The results given in Chapter II indicate that the reaction mechanism of arginine kinase from P. longipes is sequential, and that it is close to rapid equilibrium. As discussed by Gulbinsky and Cleland (1968), it had been accepted until recently that the initial velocity equation for a sequential mechanism (equation II.4.a) applies only for ordered mechanisms, or for random mechanisms in which the interconversion of the ternary complexes is the rate-limiting step. However, these authors state that the fact that initial velocity data is consistent with this equation, so that linear double reciprocal plots of initial velocity against substrate concentration appear to be obtained, does not rule out the possibility that the mechanism may be random, without the rapid equilibrium condition. The kinetic constants for a random mechanism, which is not rapid equilibrium, may be such that the non-linearity of the double reciprocal plots is so slight as to be undetectable.

The product inhibition studies reported in this chapter can be used to distinguish between a number of possible sequential mechanisms. For instance, an Ordered mechanism (Introduction, p. 6) has a product

inhibition pattern unique among those mechanisms considered; all product inhibitions are non-competitive, except that competitive inhibition occurs between the first substrate to react with the enzyme and the last product to leave (Cleland, 1963a). This mechanism, therefore, is excluded by the results obtained.

However, three reaction mechanisms give rise to an inhibition pattern composed of two competitive and two non-competitive inhibitions for each direction of the reaction. These are the rapid equilibrium, random mechanism, with two dead-end complexes, the simple ping pong and the Theorell-Chance mechanisms.

The results obtained are not consistent with a ping pong mechanism since, as can be seen by examination of such a mechanism, competitive inhibition would occur between unlike substrate-product pairs. For a Theorell-Chance mechanism, in which the steady state level of the central complexes is essentially zero, competitive product inhibition is observed both between the first substrate to add to the enzyme and the last product to dissociate, and between the second substrate to add and the first product to leave the enzyme. However, for this type of mechanism, the apparent inhibition constants derived from competitive

inhibitions, (K_{is}), do not vary with the concentration of the fixed substrate. From Table III.3, it can be seen that the K_{is} values obtained from competitive inhibition by $MgATP^{2-}$ (A) with respect to $MgADP^{-}$ (Q) differ significantly, at two different concentrations of phosphoarginine (P). Thus, it appears that the simplest mechanism consistent with the experimental data is the rapid equilibrium, random mechanism with two dead-end complexes.

On the basis of this mechanism for the arginine kinase reaction, all the values of the kinetic constants, as determined in the absence of product (Chapter II) and given in Table III.4, represent dissociation constants. Thus, K_i is the dissociation constant for the interaction of a substrate with the free enzyme and K_m, the Michaelis constant, is the dissociation constant for the interaction of a substrate with a complex of the enzyme and the other substrate of that direction of the reaction. From the values of these constants it seems that, whereas in the forward reaction $MgATP^{2-}$ combines with the enzyme and the enzyme-arginine complex similarly, and arginine also reacts equally well with the free enzyme and the enzyme-MgATP complex ($K_{ia} \approx K_a$ and $K_{ib} \approx K_b$), in the reverse reaction, the

presence of $MgADP^-$ or phosphoarginine on the enzyme hinders the combination of the other ($K_{ip} < K_p$ and $K_{iq} < K_q$). This conclusion can be reached qualitatively from the results presented in Figs. II.6 and II.7. In these initial velocity plots, the crossover point gives the dissociation constant and the intersections of the lines on the abscissa indicate the Michaelis constant.

The data from initial velocity studies in the forward direction of the reaction yield families of straight lines which intersect virtually on the abscissa, so that $K_m \approx K_i$, while those for the reverse reaction intersect below the abscissa, so that $K_i < K_m$.

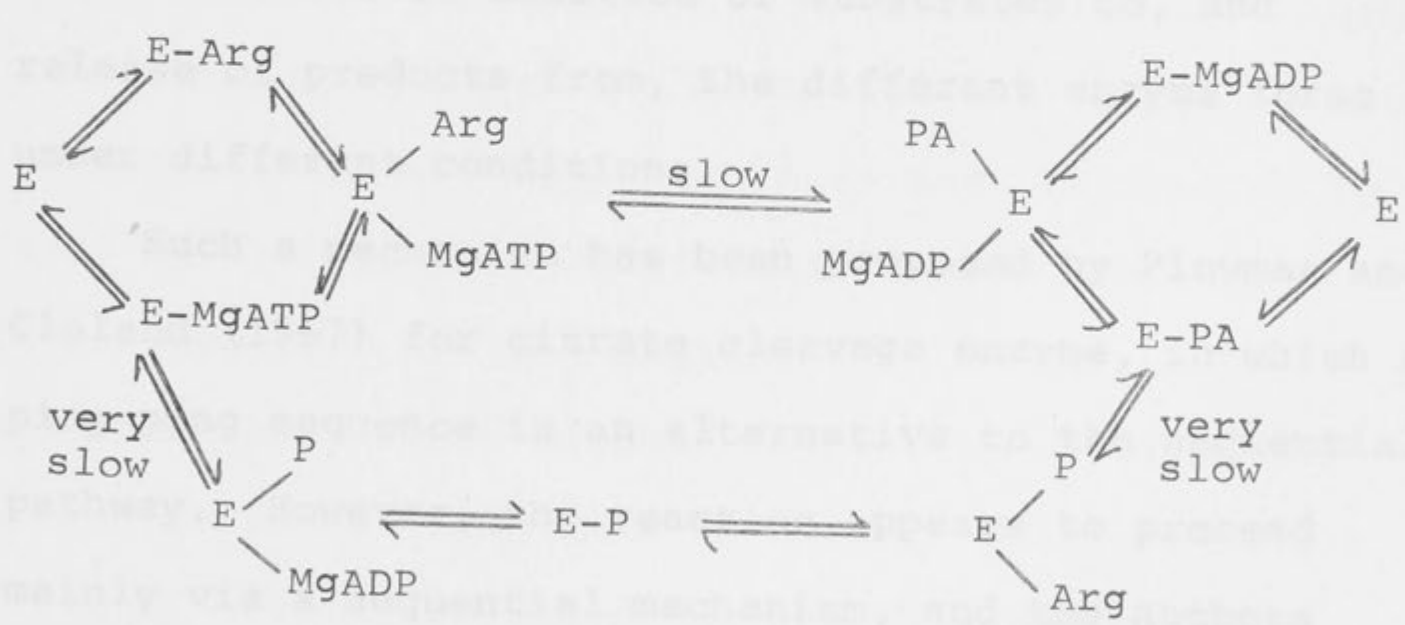
Also on the basis of this reaction mechanism, the values of the true inhibition constants were calculated, using product inhibition data, from the apparent values for these constants (Table III.3) using the relationships given in Table III.2. These values are presented in Table III.4 and, on the whole, there is satisfactory agreement between the values for the constants when they have been determined in different ways. An exception to this is the value for K_{ib}, which is significantly different when determined by initial velocity and product inhibition studies. The reason

for the discrepancy is not apparent, but similar differences have been reported by Gulbinsky and Cleland (1968) for galactokinase, which also has a random mechanism, close to being rapid equilibrium.

From the values of the kinetic constants given in Table III.4, it seems that both $MgATP^{2-}$ and $MgADP^{-}$ combine more readily with the enzyme-arginine complex than with the enzyme-phosphoarginine complex ($K_a < K_{Ia}$ and $K_{Ia} \leq K_q$). In addition, it appears that arginine combines more readily with the enzyme-MgATP complex than with the enzyme-MgADP complex ($K_{Ib} > K_b$), and that the enzyme-MgATP complex has a greater affinity for phosphoarginine than does the enzyme-MgADP complex ($K_{Ip} < K_p$). This result is unexpected, since enzyme-MgATP-phosphoarginine is a dead-end complex, whereas the enzyme-MgADP-phosphoarginine complex is an integral part of the reaction sequence.

The rapid equilibrium, random mechanism proposed for this arginine kinase does not take into account the partial exchange reactions which have been demonstrated between arginine and phosphoarginine, and between $MADP^{-}$ and $MATP^{2-}$ (Chapter II). It is possible that a very small proportion of the reaction flux can proceed by a ping pong sequence, although it is probable

that this mechanism is insignificant when both substrates are present. The ability of arginine kinase to catalyse partial exchange reactions does, nevertheless, appear to be an intrinsic property of the enzyme, and it is probable that the exchanges occur by the formation of a phosphorylated enzyme. A scheme for the reaction mechanism, omitting the dead-end complexes, could then be depicted as in Scheme III.3, where Arg, PA and P represent arginine, phosphoarginine and the phosphoryl group, respectively.



Scheme III.3

It is possible that situations such as this may occur for a number of enzymes whose mechanisms are random, but, since partial exchange reactions between like reactants are not usually sought when a reaction has

has been shown by initial velocity studies to be sequential, they would not normally be detected. Another reason for their not being detected is that, with this arginine kinase at least, relatively high concentrations of enzyme are required to demonstrate such partial exchange reactions. However, if a group, such as a phosphoryl group, is transferred to the enzyme during the overall reaction sequence, then the mechanism could be either ping pong or sequential. The dominant sequence would then be determined by the relative rates of addition of substrates to, and release of products from, the different enzyme forms under different conditions.

Such a mechanism has been proposed by Plowman and Cleland (1967) for citrate cleavage enzyme, in which a ping pong sequence is an alternative to the sequential pathway. However, the reaction appears to proceed mainly via a sequential mechanism, and the authors suggest that, under normal conditions, when $MgATP^{2-}$ and coenzyme A are present at intermediate concentrations, there would be little free phosphoenzyme formed. In the case of this arginine kinase, it seems probable that the ping pong sequence is negligible under conditions when both substrates are present at reasonable

concentrations.

The first product inhibition study involving arginine kinase was that of Griffiths *et al*. (1957), using a partially purified arginine kinase preparation from *J. verreauxi*. In this study competitive inhibition was observed with $MgADP^-$ as the product inhibitor and $MgATP^{2-}$ as the variable substrate. The concentration of $MgCl_2$ was not varied in this experiment, but was sufficiently high for the nucleotides to exist in the form of their Mg-complexes. The exact concentrations of the Mg-nucleotides, and of the free Mg^{2+} were not controlled, so that the results are difficult to interpret, since there could have been inhibition by free Mg^{2+}. However, the results appear similar to those reported in this chapter.

The only other product inhibition studies with arginine kinase that have been published involve the Type II enzyme (molecular weight 86,000) from the siponcle, *S. nudus* (Lacombe *et al*., 1969). In this study the only set of product inhibition patterns reported involved inhibition by ADP^{3-}, and the concentrations of Mg^{2+} in the reaction mixtures were equal to the ATP^{4-} concentrations, so that it would be difficult to determine the concentrations of $MgATP^{2-}$ and $MgADP^-$ present in the system.

The inhibition was competitive with respect to $MgATP^{2-}$ and uncompetitive, or possibly non-competitive with very small slope variations, with respect to arginine.

Since the results of nuclear magnetic resonance studies with the arginine kinases from H. vulgaris and H. americanus (O'Sullivan et al., 1969), and with the enzyme from P. longipes (Chapter V) indicate that the metal-nucleotide complex, rather than the free nucleotide, is the true substrate for these arginine kinases, it may well be that the same holds for the arginine kinase from S. nudus. If this is the case, no definite conclusions can be drawn from these product inhibition studies. In addition, difficulties are encountered when using ADP as a product inhibitor and $MgATP^{2-}$ as the variable substrate, because ATP is almost invariably contaminated with small amounts of ADP, and this should be taken into account when calculating the amounts of ADP required to give the desired concentrations.

Because of these factors, the results obtained from product inhibition studies with arginine kinase from S. nudus cannot be compared directly with those using the enzyme from P. longipes.

Product inhibition studies with arginine kinase from J. verreauxi have been carried out in this laboratory (Uhr, 1966) using all four reactants in turn as product inhibitors. These resulted in all non-competitive inhibitions, and the reaction mechanism of this enzyme has not been fully elucidated.

It appears from the results of the initial velocity, isotope exchange and product inhibition studies on the arginine kinase from P. longipes, that this enzyme has a rapid equilibrium, random mechanism, with two dead-end complexes, with a very minor ping pong sequence as shown in Scheme III.3.

SUMMARY

1. Initial velocity studies in the presence of the products, phosphoarginine, $MgATP^{2-}$ and $MgADP^{-}$, were carried out with the arginine kinase from P. longipes.

2. The inhibition pattern consisted of linear competitive inhibitions between phosphoarginine and arginine, $MgATP^{2-}$ and $MgADP^{-}$, and arginine and phosphoarginine, and linear non-competitive inhibitions between phosphoarginine and $MgATP^{2-}$, $MgATP^{2-}$ and phosphoarginine, and arginine and $MgADP^{-}$.

3. The inhibition patterns are consistent with a rapid equilibrium, random mechanism, with two dead-end complexes.

4. The mechanism proposed also allows for a very minor part of the reaction flux to proceed via a ping pong mechanism.

CHAPTER IV

Nucleoside Diphosphate Analogues of $MgADP^-$ as Substrates in the Reverse Direction of the Arginine Kinase Reaction, and the Effects of Dead-End Inhibitors on the Reaction

INTRODUCTION

An investigation of the ability of substrate analogues to function in an enzymic reaction as substrates or inhibitors, or both, can be used to deduce some of the features which are essential for the activity of a compound as a substrate. Also, such studies may indicate which groups on the substrate are important in determining the specificity of the enzyme for that particular substrate, and may, therefore, give an indication of the nature of the active centre of the enzyme.

The only reported studies of the specificity of arginine kinase for its substrates are those of O'Sullivan *et al.* (1969), in which $MndADP^-$, as well as $MnADP^-$, was used as a substrate for the arginine kinases of *H. vulgaris* and *H. americanus*. James and Morrison (1966a) have studied the effects of nucleotide substrate analogues on the reverse reaction of creatine kinase, and have also investigated the interaction of analogues of the phosphorylated guanidino substrates with creatine kinase (James and Morrison, 1966b). The results of the former study by James and Morrison showed that all the nucleotides tested could function as substrates of the reaction, and kinetic

constants were determined for the interaction of these compounds with the enzyme. $MgGDP^-$, which was the least effective nucleotide substrate of those tested, had a maximum velocity of 6.64% of that with $MgADP^-$ as the substrate. Of the phosphorylated guanidine substrate analogues tested, however, only phosphoglycocyamine could function as a substrate; in addition, the maximum velocity of the reaction was only 0.18% of the maximum velocity with phosphocreatine as the substrate. Phosphoarginine and phosphotaurocyamine, as well as phosphoglycocyamine, were found to be inhibitors of the reaction.

From the kinetic constants obtained, the authors concluded that the results of both studies are consistent with conformational changes occurring at the active site of creatine kinase when substrates combine with the enzyme.

In the present study of the effects of various compounds on the arginine kinase reaction, the nucleotide analogues of $MgADP^-$ used as substrates of the reverse reaction were $MgdADP^-$, $MgIDP^-$, $MgGDP^-$ and $MgUDP^-$. $MgGDP^-$, $MgUDP^-$ and $MgCDP^-$, as well as $MgPP_i^{2-}$, AMP and phosphocreatine, were used as inhibitors of the reverse reaction, and creatine was used as an

inhibitor of the forward reaction. Substrate inhibition by phosphoarginine of the reverse reaction of arginine kinase from J. verreauxi has been reported by Uhr et al. (1966), and this phenomenon was also investigated with the enzyme from P. longipes.

EXPERIMENTAL PROCEDURE

Materials

The nucleoside diphosphates, dADP, IDP, GDP, UDP and CDP were obtained from P-L Biochemicals in the form of the sodium salts, and were used without further purification after checking that they were not contaminated with other nucleotides. This was done by chromatography on DEAE-cellulose paper and the nucleotides were detected by their absorption of ultraviolet light (Morrison, 1968). Stock solutions were adjusted to pH 7.6 and stored at -10°. Inorganic pyrophosphate was Analar grade from British Drug Houses Ltd., and AMP was obtained from P-L Biochemicals. Creatine was obtained from Fluka and twice recrystallised, and phosphocreatine was prepared by the method of Ennor and Stocken (1948) as modified by Peanasky et al. (1957). Solutions of phosphocreatine were treated with Chelex 100 (Na^+ form, 200-400 mesh) from Bio-Rad Laboratories, Richmond, Cal., U.S.A., as described by Morrison and O'Sullivan (1965). L-Canavanine.H_2SO_4 was obtained from Calbiochem and used without further purification.

All other reagents were as described in Chapters I and II.

Methods

Measurement of initial velocities

Initial velocities of the reverse direction of the arginine kinase reaction were measured as described in Chapter II (p. 54), by determination of the release of arginine from phosphoarginine. Two time periods were chosen, depending on the initial rate of the reaction, to ensure that the initial velocities measured were linear with time. The amount of enzyme added was also varied : in the initial velocity studies using $MgdADP^-$ and $MgIDP^-$ as substrates, the reaction was started by the addition of 2 µg of enzyme and, with $MgGDP^-$ and $MgUDP^-$ as substrates, about 20 µg of enzyme was added. When the substrate analogues were used as inhibitors with $MgADP^-$ as the substrate, about 0.2 µg of arginine kinase was required in all cases to obtain suitable initial rates of reaction, as in Chapters II and III. All kinetic studies were carried out at 30° in 0.1 M triethanolamine buffer, pH 8.0, with 0.1 mM EDTA present, and in all cases the Mg complex of the nucleoside diphosphate was assumed to be the substrate or inhibitor. The concentration of free Mg^{2+} was held constant at 1.0 mM and the stability constant for all the Mg-nucleoside diphosphate complexes

was taken to be the same (Walaas, 1958) as for $MgADP^-$ ($4{,}000\ M^{-1}$). When inorganic pyrophosphate was used as an inhibitor it was assumed that it existed only in the form of its Mg-complex, because of its high stability constant of $250{,}000\ M^{-1}$ (Bock, 1960), and equimolar concentrations of Mg^{2+} and PP_i^{4-} were added to the reaction mixture. AMP^{2-} was considered to act as an inhibitor in the free form, and the amount of MgAMP was calculated using the stability constant $89\ M^{-1}$ (Walaas, 1958). The stability constant used for Mg-phosphocreatine was $12\ M^{-1}$ (O'Sullivan and Perrin, 1964) and free phosphocreatine was considered to be the inhibitory species. Initial velocities in the forward direction of the reaction were determined by measurement of the rate of release of ADP, which was assayed as described previously (Chapter I).

Analysis of results

The kinetic data was analysed as described in Chapter II, using the computer programmes SEQUEN, COMP, NONCOMP, PARACOMP and SUBINH (Preface, equations 5, 6, 7, 8 and 9). In all cases, the lines in the double reciprocal plots were drawn using the apparent kinetic constants obtained from computer analysis, and the points were experimentally determined. Weighted

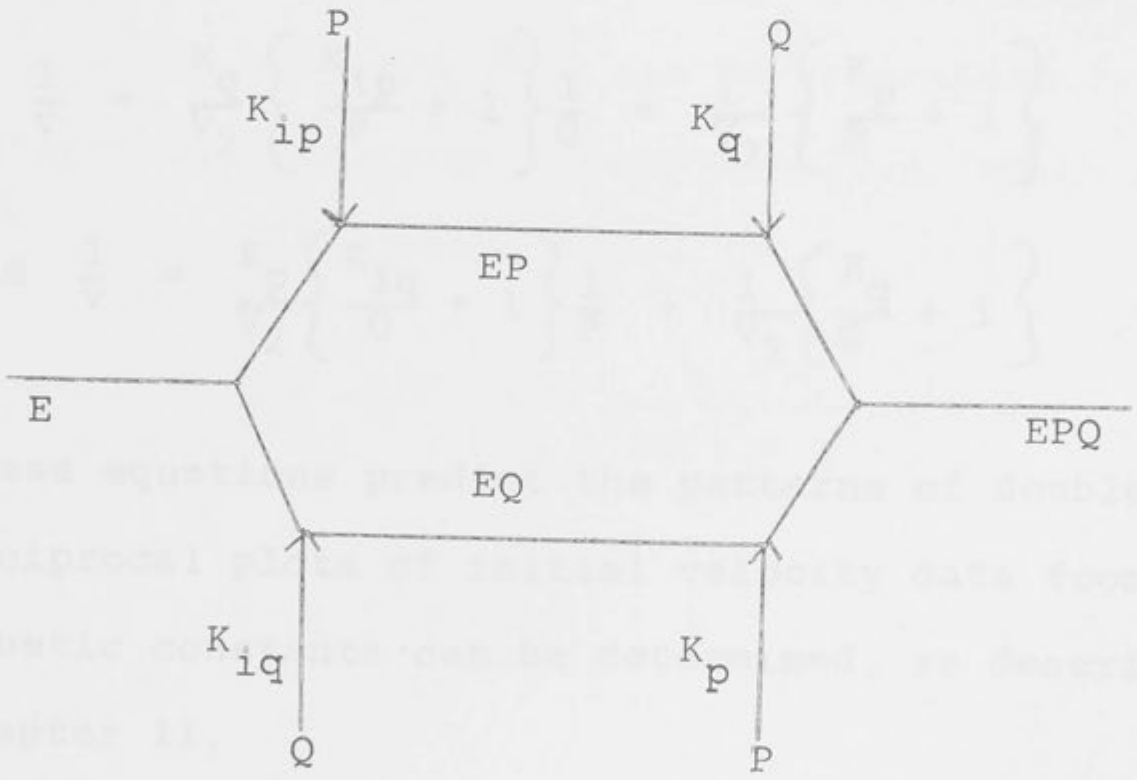
mean values for the constants, and their standard errors, were calculated using the formulae given in Chapter II.

Determination of optical rotation of D- and L-arginine

The optical rotations of solutions of D- and L-arginine were determined by measuring the rotation at a number of wavelengths and plotting the angle of rotation against $^{1}/_{\lambda}2$, where λ is the wavelength of light used (Meister, 1965, p.141). The D-arginine preparation was found to contain not more than 1.5% L-arginine.

THEORY

As discussed in Chapter III, the reaction mechanism of arginine kinase from P. longipes appears to be rapid equilibrium, random, with two dead-end complexes. As with creatine kinase (James and Morrison, 1966a), it is assumed that the reaction mechanism is not altered when the normal substrate, $MgADP^-$, is replaced by an analogue of the nucleotide substrate. The mechanism for the formation of the ternary complex is then :-



where P represents phosphoarginine, and Q the Mg-nucleotide complex. Thus, as discussed in Chapter III, the initial velocity equation is :-

$$v = \frac{V_2}{\frac{K_{iq}K_p}{PQ} + \frac{K_q}{Q} + \frac{K_p}{P} + 1} \qquad \text{(IV.1)}$$

where v is the initial velocity, V_2 is the maximum velocity, K_p and K_q are the Michaelis constants and K_{iq} is the dissociation constant for the interaction of Q with the free enzyme. Because of the rapid equilibrium condition, the relationship, $K_{ip}K_q = K_{iq}K_p$, holds, where K_{ip} is the dissociation constant for the interaction of P with the free enzyme.

In double reciprocal form equation IV.1 gives the equations :-

$$\frac{1}{v} = \frac{K_q}{V_2}\left\{\frac{K_{ip}}{P} + 1\right\}\frac{1}{Q} + \frac{1}{V_2}\left\{\frac{K_p}{P} + 1\right\} \qquad \text{(IV.2.a)}$$

and $$\frac{1}{v} = \frac{K_p}{V_2}\left\{\frac{K_{iq}}{Q} + 1\right\}\frac{1}{P} + \frac{1}{V_2}\left\{\frac{K_q}{Q} + 1\right\} \qquad \text{(IV.2.b)}$$

These equations predict the patterns of double reciprocal plots of initial velocity data from which kinetic constants can be determined, as described in Chapter II.

Effect of substrate analogues as inhibitors

A substrate analogue is likely to inhibit the reaction by combination at the site at which the substrate would normally react. If an analogue (N) of the nucleotide substrate (Q) is the inhibitor, then it is assumed to combine both with the free

enzyme and with the enzyme-phosphoarginine complex, so that the initial velocity equation becomes :-

$$v = \frac{V_2}{\frac{K_{iq}K_p}{PQ}\left\{1 + \frac{N}{K_{in}}\right\} + \frac{K_p}{P} + \frac{K_q}{Q}\left\{1 + \frac{N}{K_{In}}\right\} + 1} \qquad \text{(IV.3)}$$

where K_{in} and K_{In} are the dissociation constants for the interaction of N with the free enzyme and with the enzyme-phosphoarginine complex, respectively.

With phosphoarginine, (P), as the variable substrate, equation IV.3 can be rearranged in double reciprocal form to give equation IV.4, which predicts non-competitive inhibition.

$$\frac{1}{v} = \frac{K_p}{V_2}\left\{\left[1 + \frac{K_{iq}}{Q}\right]\left[1 + \frac{N}{K_{in}\left(1 + \frac{Q}{K_{iq}}\right)}\right]\right\}\cdot\frac{1}{P} + \frac{1}{V_2}\left\{\left[1 + \frac{K_q}{Q}\right]\left[1 + \frac{N}{K_{In}\left(1 + \frac{Q}{K_q}\right)}\right]\right\} \qquad \text{(IV.4)}$$

The true values of K_{in} and K_{In} can be calculated from the non-competitive inhibitions using the apparent values obtained by replotting slopes and intercepts against inhibitor concentrations :-

$$\text{Apparent } K_{in} = K_{in}\left(1 + \frac{Q}{K_{iq}}\right)$$

$$\text{Apparent } K_{In} = K_{In}\left(1 + \frac{Q}{K_q}\right)$$

Equation IV.3 can also be rearranged in double reciprocal form with $MgADP^-$ (Q) as the variable substrate, in which case equation IV.5, predicting competitive inhibition, is obtained :-

$$\frac{1}{v} = \frac{K_q}{V_2}\left\{\left[1 + \frac{K_p}{P}\right]\left[1 + \frac{N}{\left(\frac{K_{ip}}{K_{in}P} + \frac{1}{K_{In}}\right)}\right]\right\}\frac{1}{Q} + \frac{1}{V_2}\left\{1 + \frac{K_p}{P}\right\} \qquad (IV.5)$$

By replotting the slopes of the competitive inhibition predicted by equation IV.5 against inhibitor concentration, the value of the complex function involving both K_{in} and K_{In}, as well as K_{ip} and P is obtained :-

$$\text{Apparent } K_i = \frac{\left(1 + \frac{K_{ip}}{P}\right)}{\left(\frac{K_{ip}}{K_{in}P} + \frac{1}{K_{In}}\right)}$$

The value obtained experimentally for this constant can be compared with that calculated by substituting values for the constants K_{ip}, K_{in} and K_{In} which were obtained previously, and for P, the fixed concentration of phosphoarginine used.

In the case where a Mg-nucleotide can be studied both as a substrate of the reaction, and as an inhibitor of the reaction with $MgADP^-$ as substrate, the dissociation constants for the interaction of the compound with the free enzyme should be the same whichever method is used ($K_{iq} = K_{in}$). Similarly, the dissociation constants determined by the two methods for the interaction of the compound with the enzyme-phosphoarginine complex should be the same ($K_q = K_{In}$).

RESULTS

Initial velocity studies of the reverse direction of the arginine kinase reaction with Mg-nucleotide substrate analogues

Initial velocity experiments were carried out with $MgdADP^-$, $MgIDP^-$, $MgUDP^-$ and $MgGDP^-$ as substrates and the initial velocity patterns obtained are shown in Figs. IV.1-4. The initial velocities of the reactions are very much lower than when the normal substrate, $MgADP^-$, is used. Also, the concentrations of the Mg-nucleotide complexes used in these studies are considerably higher than the concentrations of $MgADP^-$ normally employed. The initial velocity patterns in all cases consist of families of converging lines, although the slope changes observed when $MgIDP^-$ and $MgGDP^-$ are substrates are relatively small.

The data from these plots was analysed using the SEQUEN computer programme, and the results are given in Table IV.1. The values are the weighted means of constants obtained from at least two experiments in each case, and the values obtained with $MgADP^-$ and reported in Chapter II are included for comparison. The maximum velocities are shown, as well as the Michaelis and dissociation constants for the Mg-

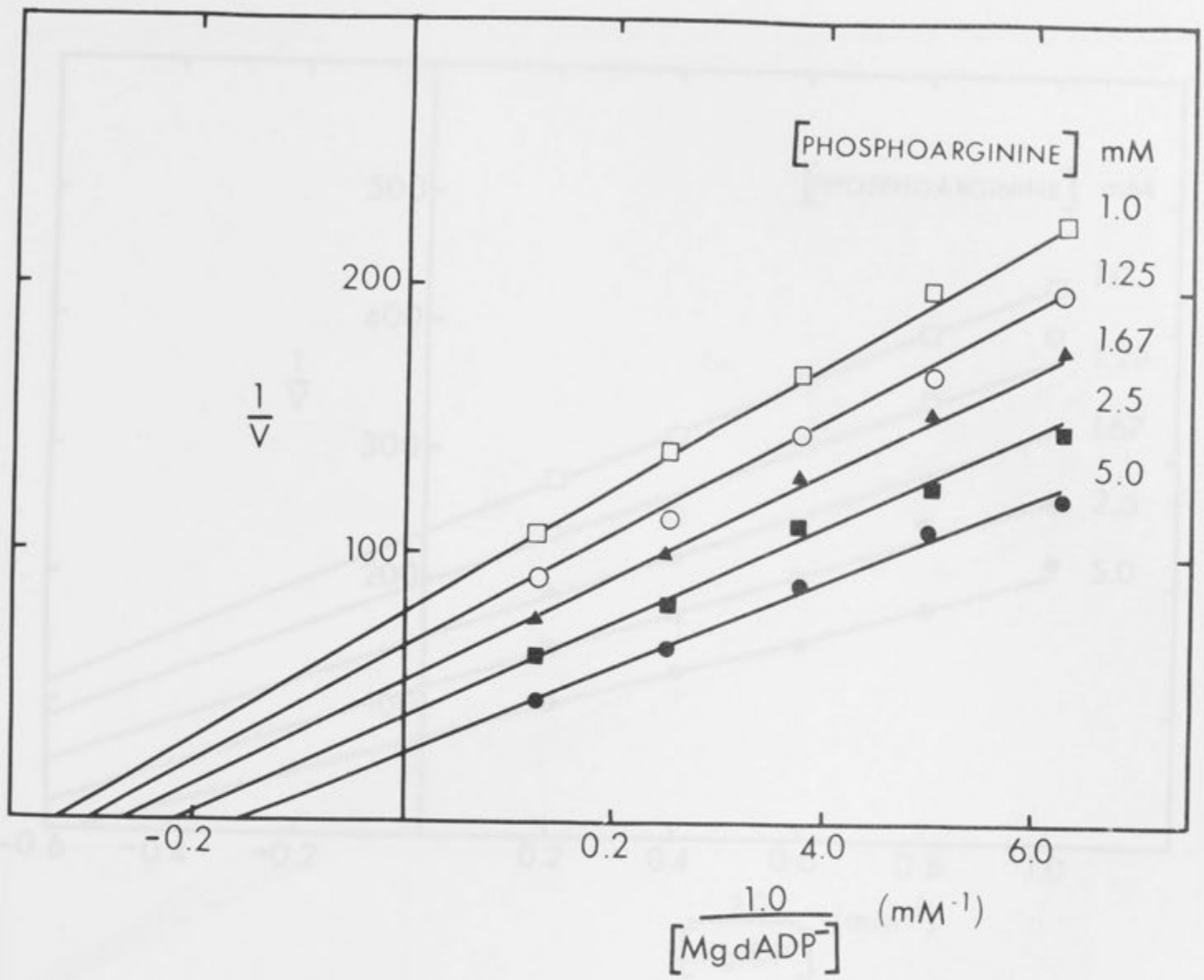


Fig. IV.1. Effect of phosphoarginine on the initial velocity of the reverse reaction with MgdeoxyADP$^-$ as the variable substrate. Velocities are expressed as µmoles per min per µg of arginine kinase.

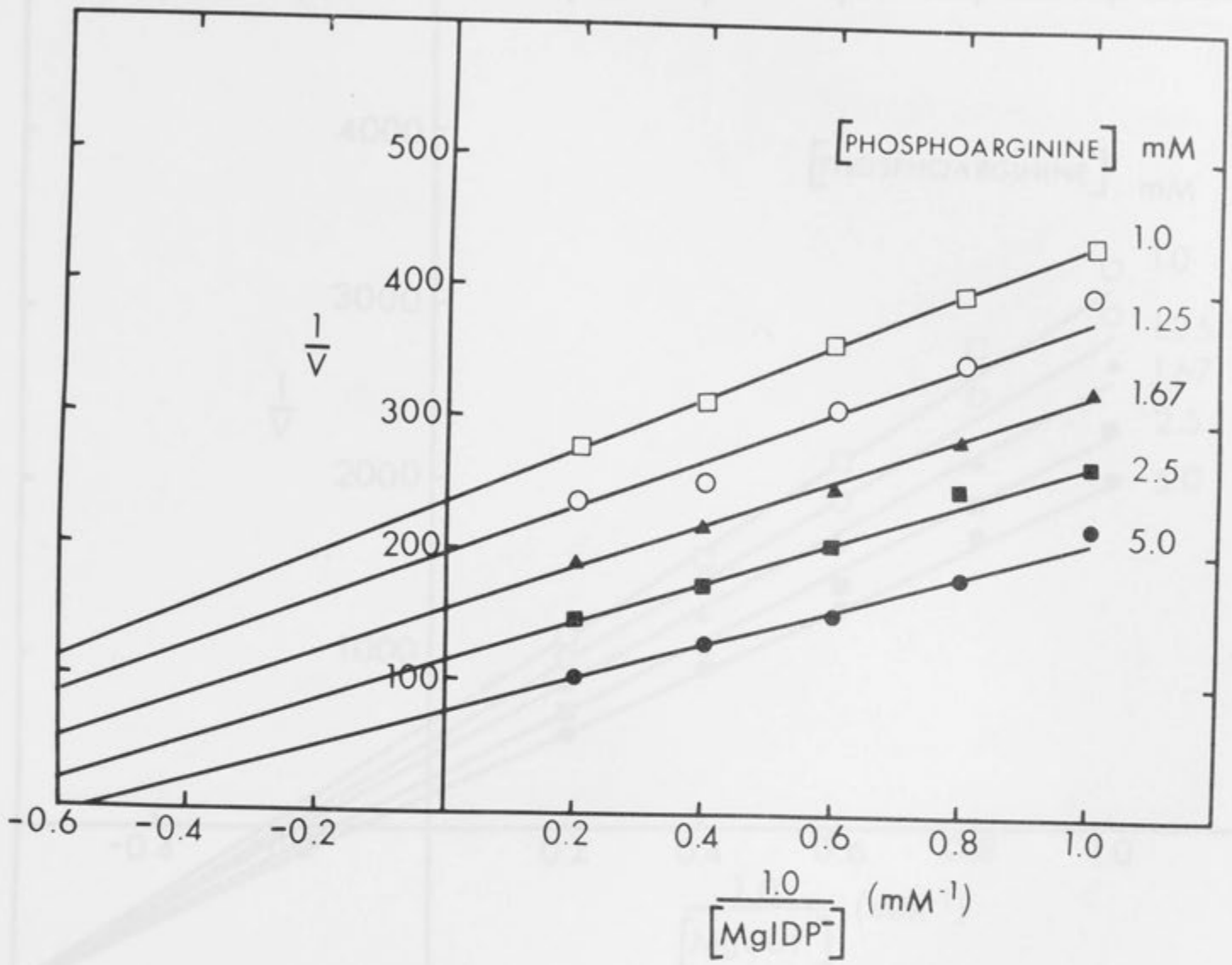


Fig. IV.2. Effect of phosphoarginine on the initial velocity of the reverse reaction with $MgIDP^-$ as the variable substrate. Velocities are expressed as µmoles per min per µg of arginine kinase.

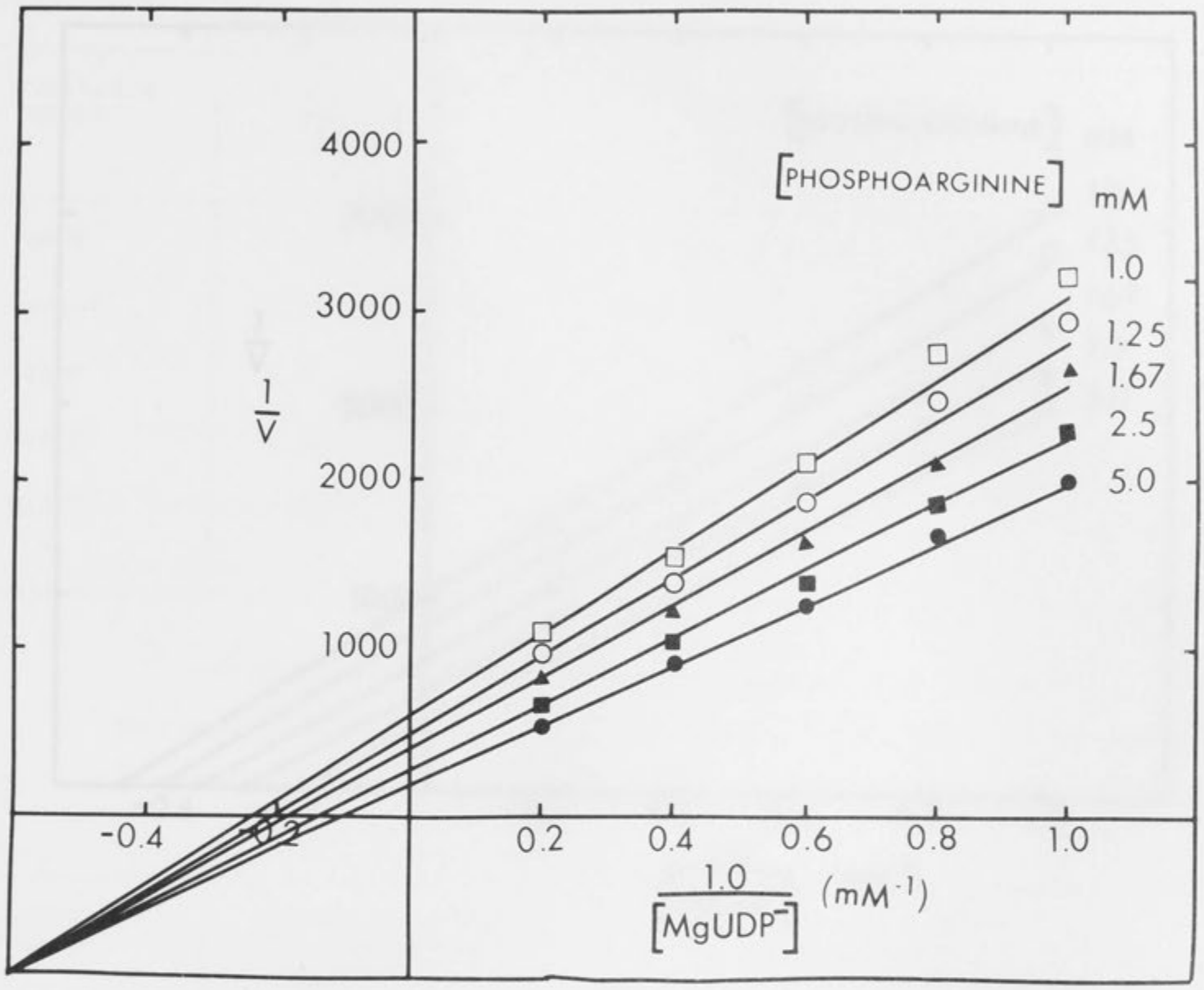


Fig. IV.3. Effect of phosphoarginine on the initial velocity of the reverse reaction with $MgUDP^-$ as the variable substrate. Velocities are expressed as μmoles per min per μg of arginine kinase.

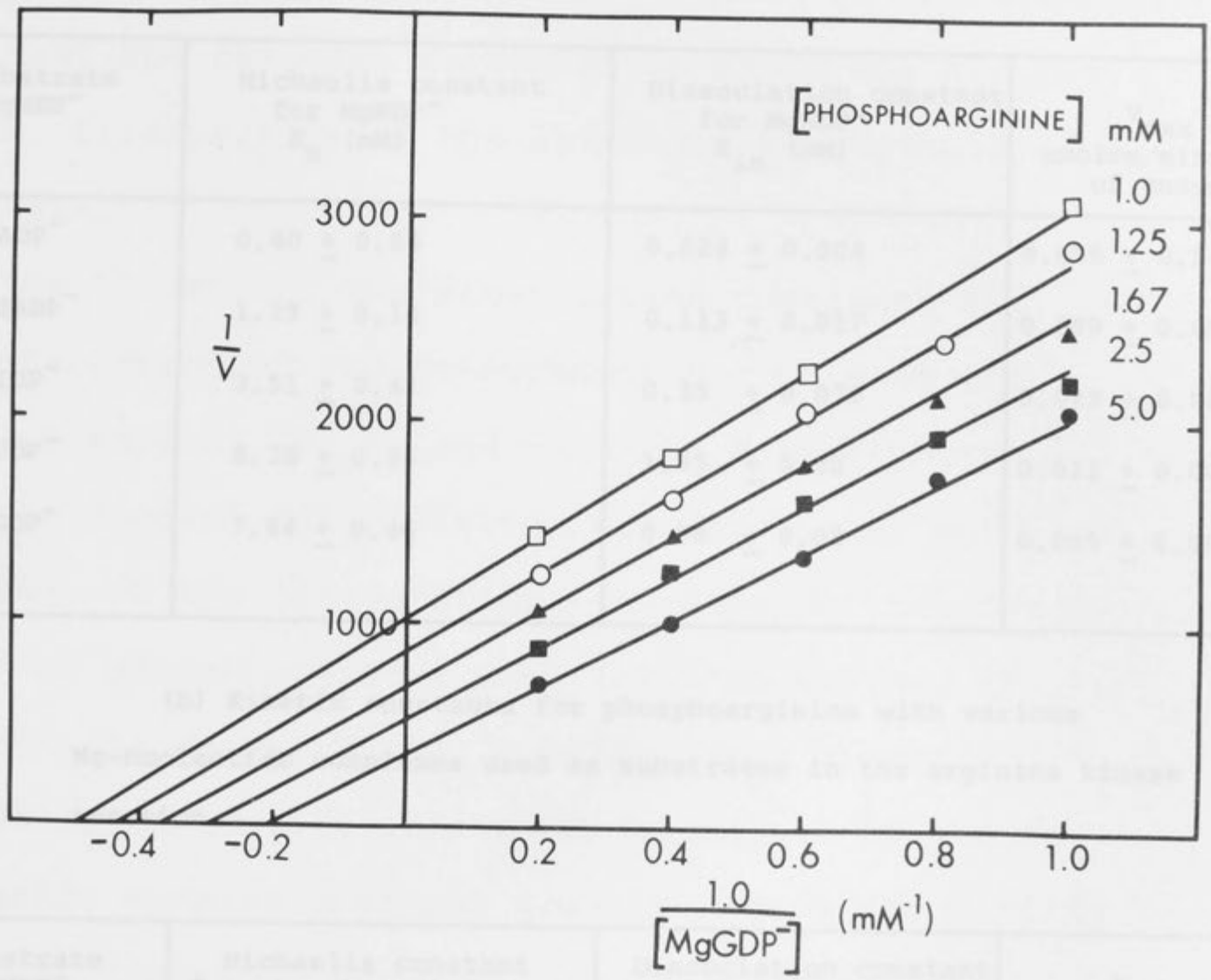


Fig. IV.4. Effect of phosphoarginine on the initial velocity of the reverse reaction with $MgGDP^-$ as the variable substrate. Velocities are expressed as μmoles per min per μg of arginine kinase.

Table IV.1.

(a) Kinetic constants for various Mg-nucleotide complexes when used as substrates in the arginine kinase reaction.

Substrate $MgNDP^-$	Michaelis constant for $MgNDP^-$ K_n (mM)	Dissociation constant for $MgNDP^-$ K_{in} (mM)	V_{max} µmoles/min/µg of enzyme
$MgADP^-$	0.40 ± 0.04	0.024 ± 0.004	0.606 ± 0.046
$MgdADP^-$	1.29 ± 0.14	0.113 ± 0.017	0.089 ± 0.007
$MgIDP^-$	3.51 ± 0.41	0.35 ± 0.079	0.029 ± 0.002
$MgUDP^-$	8.36 ± 0.96	1.95 ± 0.20	0.011 ± 0.0002
$MgGDP^-$	7.84 ± 0.46	0.58 ± 0.08	0.005 ± 0.0002

(b) Kinetic constants for phosphoarginine with various Mg-nucleotide complexes used as substrates in the arginine kinase reaction.

Substrate $MgNDP^-$	Michaelis constant for phosphoarginine K_p (mM)	Dissociation constant for phosphoarginine K_{ip} (mM)	V_{max} µmoles/min/µg of enzyme
$MgADP^-$	3.82 ± 0.39	0.26 ± 0.04	0.606 ± 0.046
$MgdADP^-$	5.97 ± 0.68	0.51 ± 0.08	0.089 ± 0.007
$MgIDP^-$	5.93 ± 0.61	0.50 ± 0.12	0.029 ± 0.002
$MgUDP^-$	2.64 ± 0.39	0.68 ± 0.05	0.011 ± 0.0002
$MgGDP^-$	4.01 ± 0.27	0.30 ± 0.04	0.005 ± 0.0002

nucleotide ($MgNDP^-$) complexes, and for phosphoarginine in the presence of these substrate analogues. The values for these constants are all based on the assumption that the reaction mechanism is rapid equilibrium, random, irrespective of the nature of the Mg-nucleotide substrate.

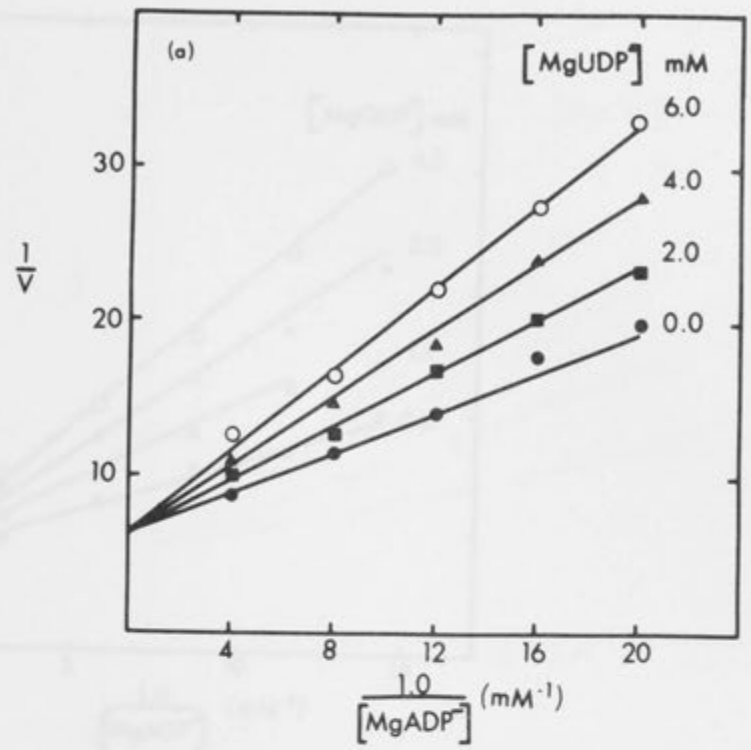
The values for the dissociation constants for the interaction of the Mg-nucleotide complexes with the free enzyme, (K_{in}), and with the enzyme-phosphoarginine complex, (K_n) (Table IV.1.(a)), vary much more than do the dissociation constants for the interaction of phosphoarginine with the free enzyme, (K_{ip}), and with the enzyme-MgNDP complex, (K_p) (Table IV.1.(b)). This indicates that the nature of the Mg-nucleotide complex does not greatly affect the binding of phosphoarginine, but that the structure of the nucleotide does alter its ability to combine at the active site, both in the absence and presence of phosphoarginine.

Inhibition studies with $MgUDP^-$, $MgGDP^-$ and $MgCDP^-$

Because $MgUDP^-$ and $MgGDP^-$ are such poor substrates for the arginine kinase reaction, they can, under appropriate conditions, be used as inhibitors without complications caused by their substrate

activity. $MgCDP^-$ is also a substrate for the reaction but, because the initial velocities of the reaction in the presence of this compound were so low, it proved impracticable to use it in initial velocity experiments. $MgCDP^-$ was, however, an effective inhibitor of the enzymic reaction. When these Mg-nucleotides were used as inhibitors of the reaction, the enzyme concentration was very low, 0.2 µg, as compared with 20 µg/ml used to demonstrate substrate activities, so that their activities as substrates were insignificant. Nevertheless, controls were included in all experiments, with the highest concentration of the inhibitor used and all reactants except $MgADP^-$ present. No reaction could be detected in the controls.

The results of these dead-end inhibition studies are shown in Figs. IV.5-7, in the form of double reciprocal plots. All the Mg-nucleotide complexes give competitive inhibition with respect to $MgADP^-$, and non-competitive inhibition with respect to phosphoarginine, as would be expected if the reaction mechanism is rapid equilibrium, random, with two dead-end complexes. Assuming this to be the reaction mechanism, the values obtained for the true dissociation constants, (K_{iq} and K_q), from initial velocity studies



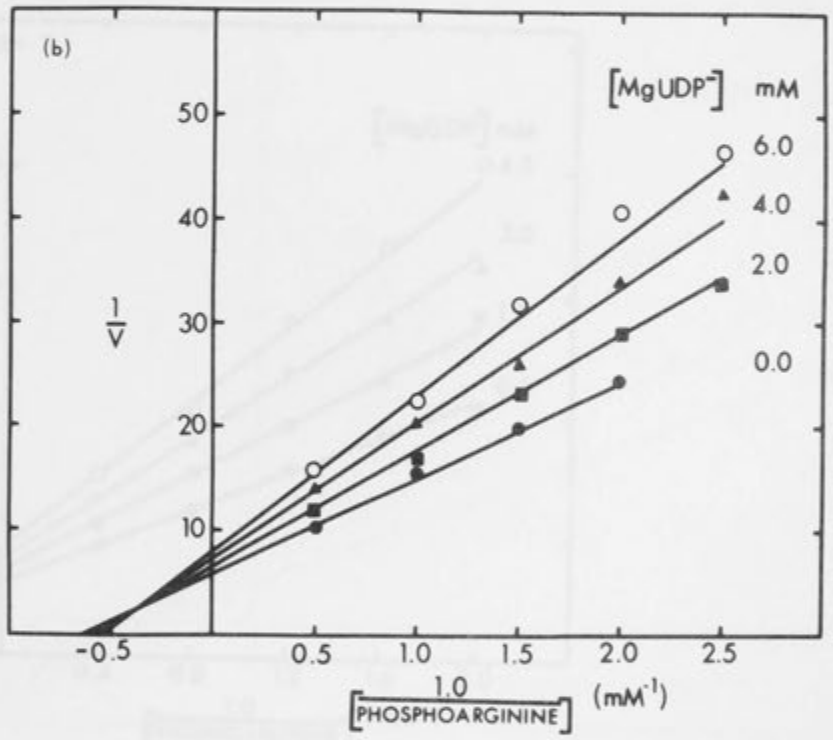
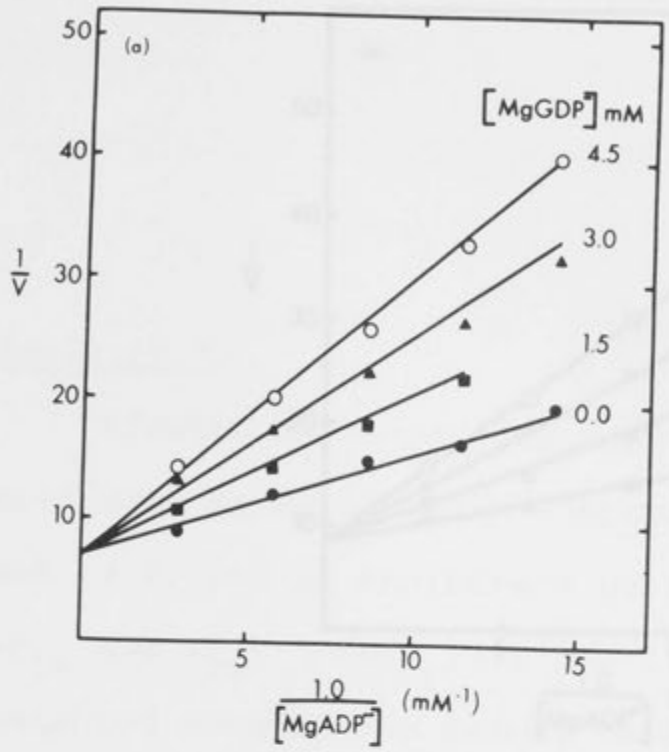


Fig. IV.5. Inhibition of the reverse reaction by $MgUDP^-$, (a), with $MgADP^-$ the variable substrate and phosphoarginine held constant at 1.25 mM, and, (b), with phosphoarginine the variable substrate and $MgADP^-$ held constant at 0.1 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.



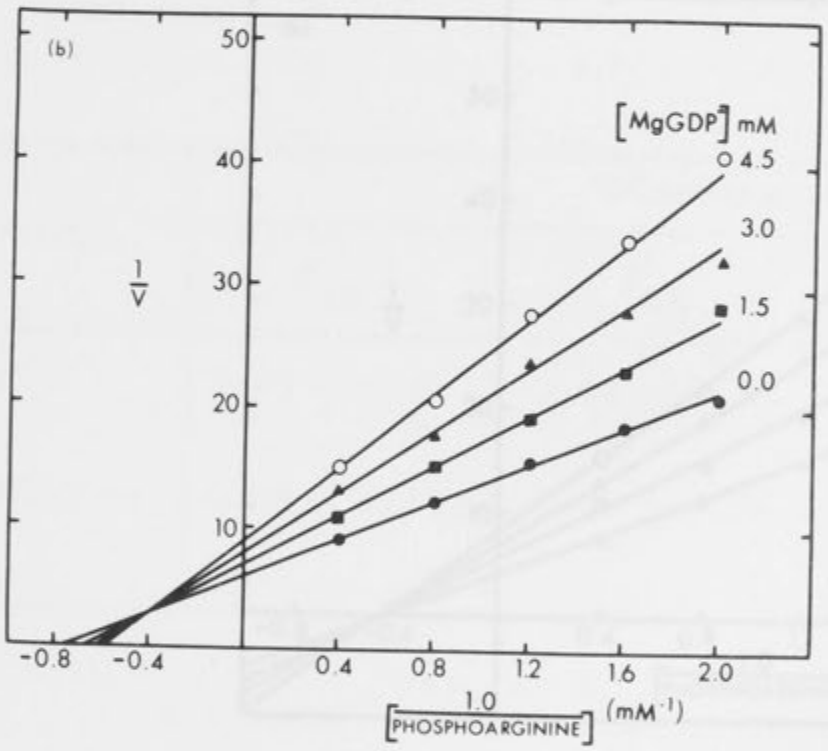


Fig. IV.6. Inhibition of the reverse reaction by $MgGDP^-$, (a), with $MgADP^-$ as the variable substrate and phosphoarginine held constant at 1.25 mM, and, (b), with phosphoarginine as the variable substrate and $MgADP^-$ held constant at 0.125 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.

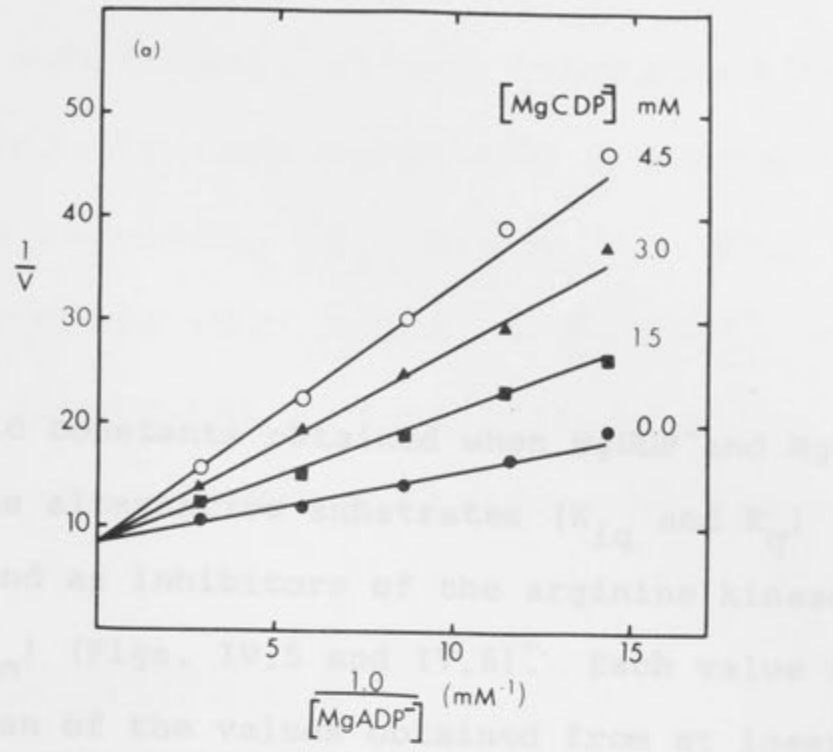


Fig. IV.7. Inhibition of the reverse reaction by $MgCDP^-$, (a), with $MgADP^-$ as the variable substrate and phosphoarginine held constant at 1.25 mM, and, (b), with phosphoarginine as the variable substrate and $MgADP^-$ held constant at 0.125 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.

Table IV.2.

Kinetic constants obtained when $MgUDP^-$ and $MgGDP^-$ are used both as alternative substrates (K_{iq} and K_q) (Figs. IV.3 and IV.4) and as inhibitors of the arginine kinase reaction (K_{in} and K_{In}) (Figs. IV.5 and IV.6). Each value is the weighted mean of the values obtained from at least two experiments.

Reactant	Dissociation constant			
	K_{iq}	K_{in}	K_q	K_{In}
$MgUDP^-$	1.95 ± 0.20	2.10 ± 0.45	8.36 ± 0.96	5.35 ± 1.13
$MgGDP^-$	0.58 ± 0.08	0.79 ± 0.02	7.84 ± 0.46	6.21 ± 0.15

$$E + MgNDP^- \overset{K_{iq} \text{ or } K_{in}}{\rightleftharpoons} E - MgNDP$$

$$E - PA + MgNDP^- \overset{K_q \text{ or } K_{In}}{\rightleftharpoons} E - PA - MgNDP$$

with alternative substrates, should correspond to those obtained when the same compounds are used as inhibitors of the reaction, (K_{in} and K_{In}). The results of such studies with $MgUDP^-$ and $MgGDP^-$ as both alternative substrates and inhibitors shows fair agreement between the constants obtained by the two methods (Table IV.2).

Inhibition by Mg-pyrophosphate

Inhibition of the reverse direction of the arginine kinase reaction by $MgPP_i^{2-}$ was investigated and the results are shown in Fig. IV.8. The data were fitted to the computer programme for linear competitive inhibition, COMP, and to the PARACOMP programme. Better fits were obtained when the data were analysed using the PARACOMP programme, and therefore it seems that the inhibition by $MgPP_i^{2-}$ with respect to both phosphoarginine and $MgADP^-$ is competitive, with replots of slope against inhibitor concentration resulting in parabolas. The apparent kinetic constants, K_{I1} and K_{I2}, calculated from the slope variations were obtained from the output of computer analysis of the data using the PARACOMP programme. With $MgADP^-$ as the variable substrate, K_{I1} = 10.68 $\pm$ 1.71 mM and K_{I2} = 38.18 $\pm$ 2.91 mM, and with

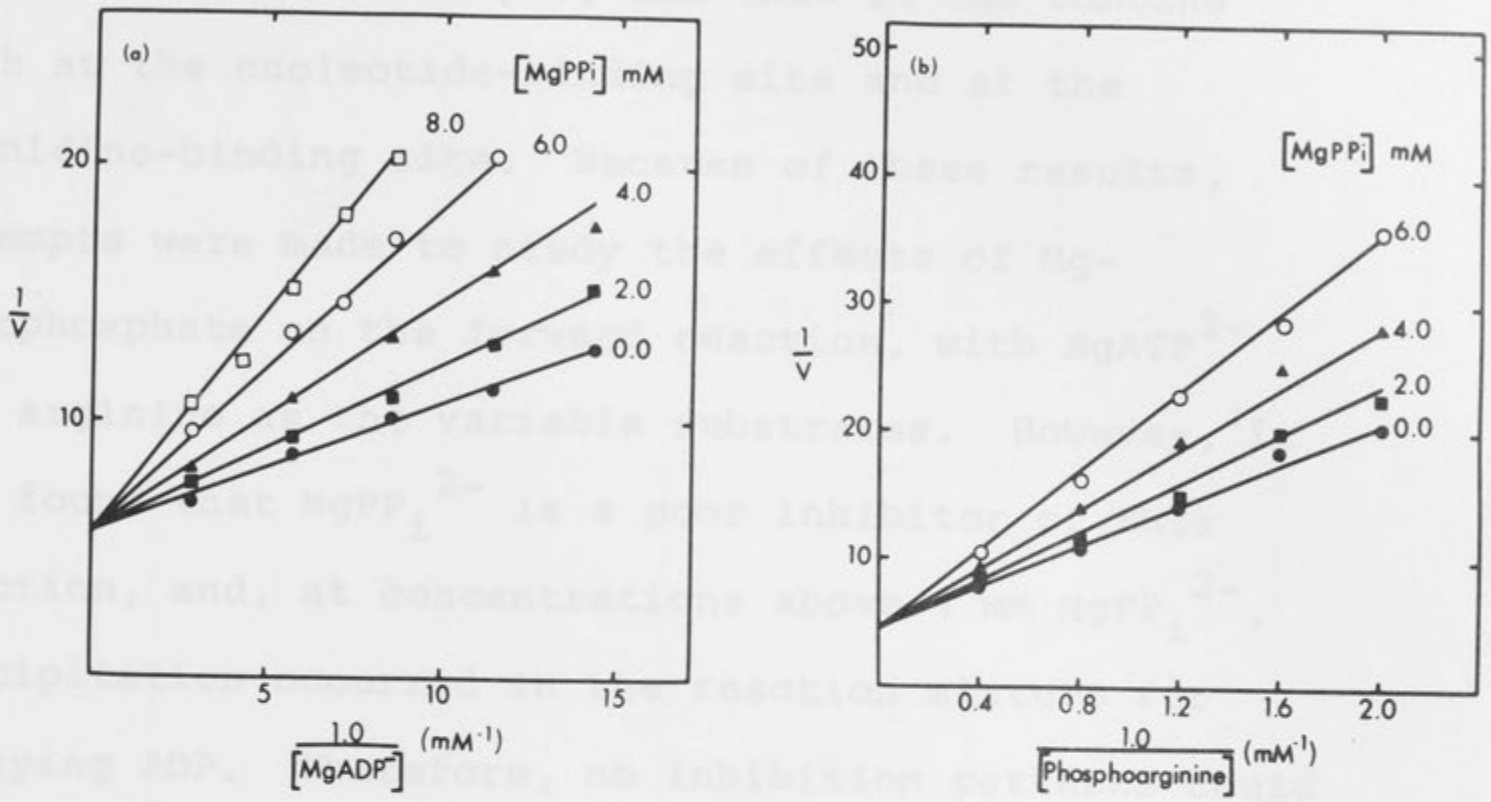


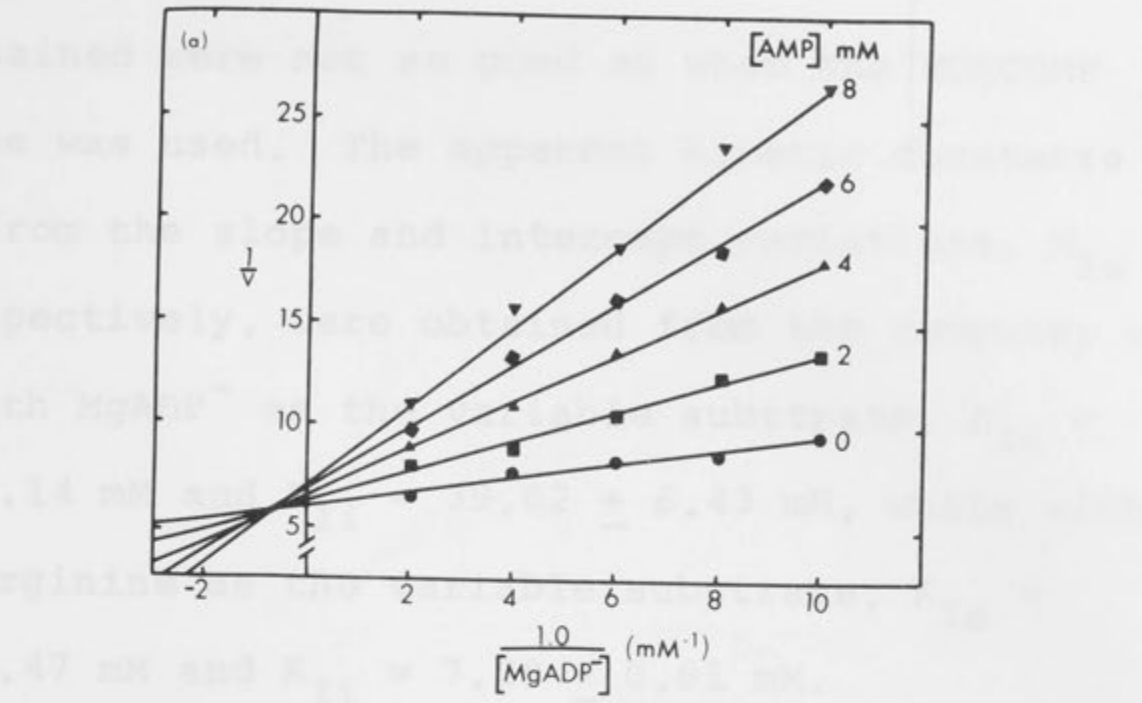
Fig. IV.8. Inhibition of the reverse reaction by $MgPP_i^{2-}$, (a) with $MgADP^-$ as the variable substrate and phosphoarginine held constant at 1.25 mM, and, (b) with phosphoarginine as the variable substrate and $MgADP^-$ held constant at 0.125 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.

phosphoarginine as the variable substrate, K_{I1} = 10.71 ± 1.76 mM and K_{I2} = 57.18 ± 5.71 mM.

It seems plausible that $MgPP_i^{2-}$ can react more than once with the enzyme, and that it can combine both at the nucleotide-binding site and at the guanidino-binding site. Because of these results, attempts were made to study the effects of Mg-pyrophosphate on the forward reaction, with $MgATP^{2-}$ and arginine as the variable substrates. However, it was found that $MgPP_i^{2-}$ is a poor inhibitor of this reaction, and, at concentrations above 4 mM $MgPP_i^{2-}$, precipitation occurred in the reaction mixture for assaying ADP. Therefore, no inhibition patterns could be obtained for the effect of $MgPP_i^{2-}$ on the forward reaction.

Inhibition by AMP^{2-}

AMP^{2-} was used as an inhibitor of the reverse reaction with respect to both $MgADP^-$ and phospho-arginine as variable substrates, and in both cases non-competitive inhibition was observed (Fig. IV.9). The data were analysed using the NONCOMP computer programme. Because of the very small intercept variation when $MgADP^-$ is the variable substrate, these data were also analysed using the COMP programme. However, the



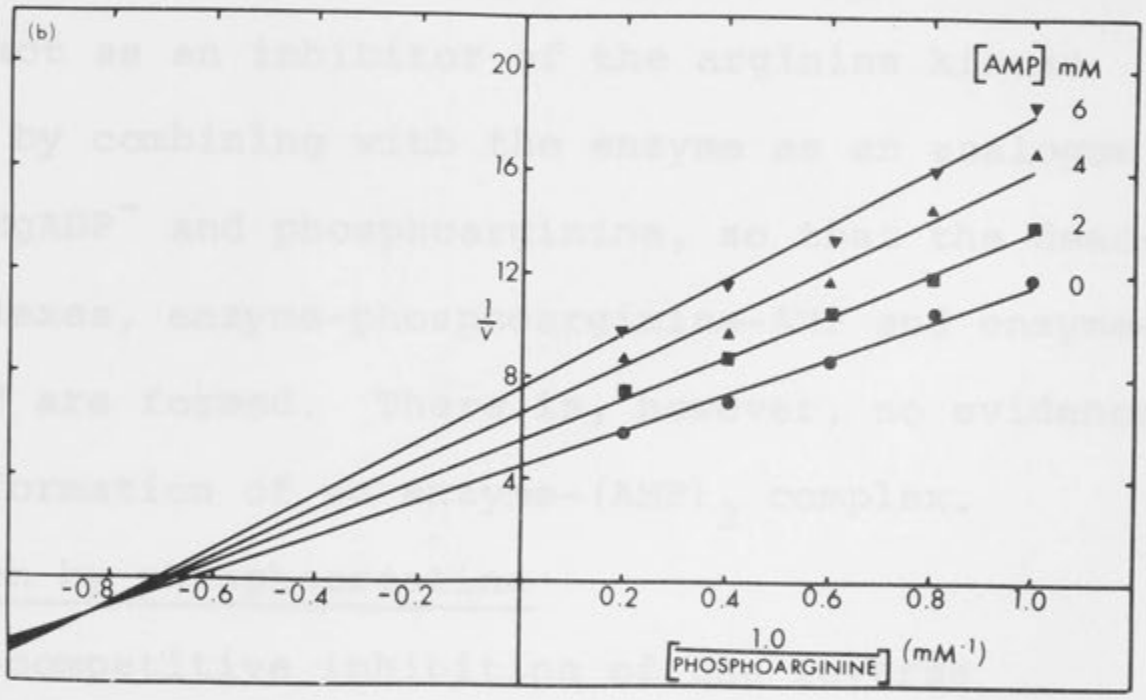


Fig. IV.9. Inhibition of the reverse reaction by AMP, (a) with $MgADP^-$ as the variable substrate and phosphoarginine held constant at 2.5 mM, and, (b) with phosphoarginine as the variable substrate and $MgADP^-$ held constant at 0.25 mM. Velocities are expressed as µmoles per min per µg of arginine kinase.

fits obtained were not so good as when the NONCOMP programme was used. The apparent kinetic constants obtained from the slope and intercept variations, K_{Is} and K_{Ii}, respectively, were obtained from the computer output. With $MgADP^-$ as the variable substrate, K_{Is} = 1.93 $\pm$ 0.14 mM and K_{Ii} = 39.02 $\pm$ 6.43 mM, while with phosphoarginine as the variable substrate, K_{Is} = 9.67 $\pm$ 1.47 mM and K_{Ii} = 7.29 $\pm$ 0.61 mM.

It seems, from the results of these studies, that AMP may act as an inhibitor of the arginine kinase reaction by combining with the enzyme as an analogue of both $MgADP^-$ and phosphoarginine, so that the dead-end complexes, enzyme-phosphoarginine-AMP and enzyme-MgADP-AMP are formed. There is, however, no evidence for the formation of an enzyme-$(AMP)_2$ complex.

Inhibition by phosphocreatine

Non-competitive inhibition of the reverse direction of the arginine kinase reaction by phosphocreatine was observed with respect to both $MgADP^-$ and phosphoarginine as variable substrates (Fig. IV.10). The data were analysed using the NONCOMP computer programme and the values for the apparent kinetic constants, K_{Is} and K_{Ii}, were obtained from the computer output. With phosphoarginine as the

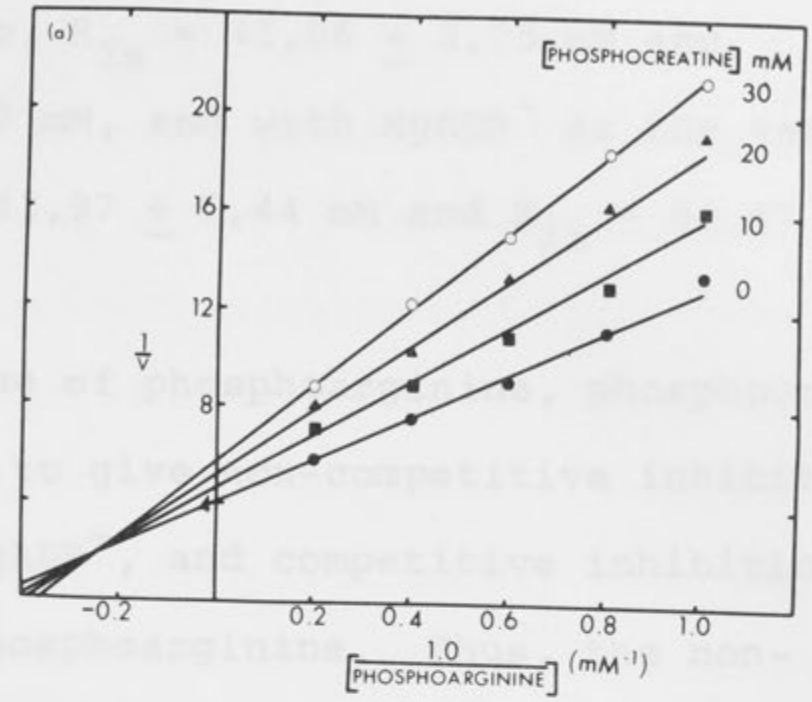


Fig. IV.10. Inhibition of the reverse reaction by phosphocreatine, (a) with phosphoarginine as the variable substrate and $MgADP^-$ held constant at 0.25 mM and, (b) with $MgADP^-$ as the variable substrate and phosphoarginine held constant at 2.5 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.

variable substrate, K_{Is} = 41.06 ± 3.75 mM and K_{Ii} = 60.65 ± 6.67 mM, and with $MgADP^-$ as the variable substrate, K_{Is} = 43.97 ± 7.44 mM and K_{Ii} = 56.47 ± 3.88 mM.

As an analogue of phosphoarginine, phosphocreatine would be expected to give non-competitive inhibition with respect to $MgADP^-$, and competitive inhibition with respect to phosphoarginine. Thus, the non-competitive inhibition observed with phosphoarginine as the variable substrate is not consistent with the proposed mechanism. However, relatively high concentrations are required to demonstrate the inhibition by phosphocreatine and this may occur in a similar manner to the substrate inhibition by phosphoarginine discussed later in this chapter.

Effect of analogues of arginine on the forward direction of the arginine kinase reaction

The analogues of L-arginine which were tested as substrates and inhibitors of the forward direction of the reaction were D-arginine, canavanine and creatine.

In order to test for substrate activity, the reaction was carried out with D-arginine at a concentration of 6 mM. This concentration may be compared with the kinetic constants for L-arginine, which are

K_{ib} = 0.81 mM and K_b = 0.75 mM (Table II.2). Eighty times the concentration of enzyme used when L-arginine was the substrate was added to the reaction mixture. The slight reaction which occurred did not increase with time, and could be attributed to slight contamination of D-arginine with L-arginine. D-Arginine did not function as an inhibitor of the reaction when present at a concentration of 6 mM with L-arginine present at 0.5 mM, indicating that any affinity of the enzyme for D-arginine could not be detected in this way.

Canavanine, at a concentration of 6 mM, and with 80 times the usual enzyme concentration, showed very slight substrate activity, and at 6 mM with L-arginine as the substrate at a concentration of 0.3 mM, caused no detectable inhibition of the reaction.

With creatine at a concentration of 6 mM and 80 times the usual arginine kinase concentration no enzymic activity could be detected. However, this guanidine was found to be a fairly effective inhibitor of the reaction, and was therefore studied as an inhibitor with arginine as the variable substrate. A competitive inhibition pattern (Fig. IV.11) was obtained.

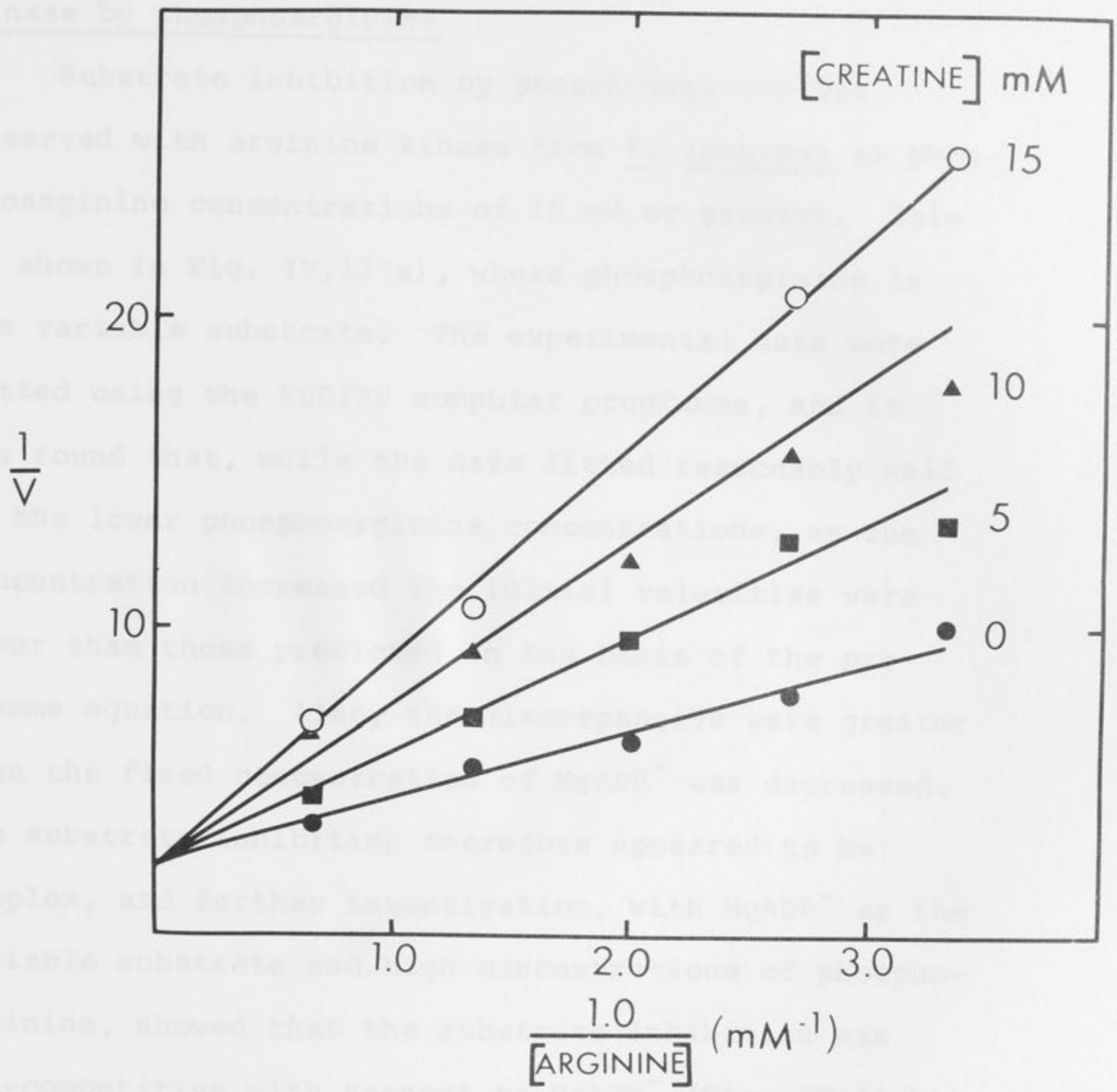


Fig. IV.11. Inhibition of the forward reaction by creatine, with arginine as the variable substrate and $MgATP^{2-}$ held constant at 1.2 mM. Velocities are expressed as μmoles per min per μg of arginine kinase.

Substrate inhibition of the reverse reaction of arginine kinase by phosphoarginine

Substrate inhibition by phosphoarginine was observed with arginine kinase from P. longipes at phosphoarginine concentrations of 10 mM or greater. This is shown in Fig. IV.12(a), where phosphoarginine is the variable substrate. The experimental data were fitted using the SUBINH computer programme, and it was found that, while the data fitted reasonably well at the lower phosphoarginine concentrations, as the concentration increased the initial velocities were lower than those predicted on the basis of the programme equation. Also, the discrepancies were greater when the fixed concentration of $MgADP^-$ was decreased. The substrate inhibition therefore appeared to be complex, and further investigation, with $MgADP^-$ as the variable substrate and high concentrations of phosphoarginine, showed that the substrate inhibition was non-competitive with respect to $MgADP^-$ (Fig. IV.12(b)).

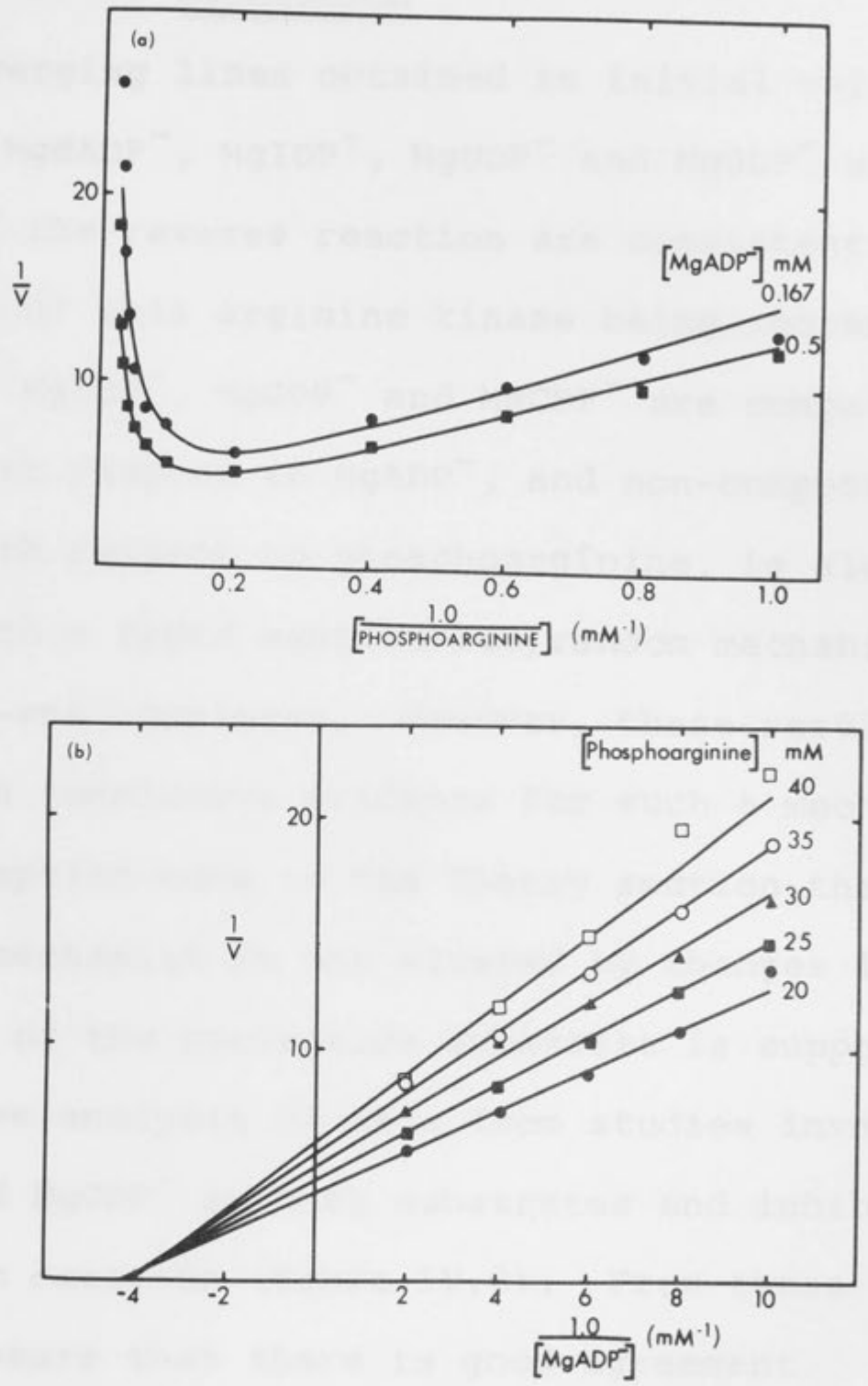


Fig. IV.12. Substrate inhibition of the reverse reaction by phosphoarginine, (a) with phosphoarginine as the variable substrate and, (b) with $MgADP^-$ as the variable substrate. Velocities are expressed as μmoles per min per μg of arginine kinase.

DISCUSSION

The converging lines obtained in initial velocity studies with $MgdADP^-$, $MgIDP^-$, $MgUDP^-$ and $MgGDP^-$ as substrates of the reverse reaction are consistent with the mechanism of this arginine kinase being sequential. The fact that $MgUDP^-$, $MgGDP^-$ and $MgCDP^-$ are competitive inhibitors with respect to $MgADP^-$, and non-competitive inhibitors with respect to phosphoarginine, is also consistent with a rapid equilibrium, random mechanism with two dead-end complexes. However, these results do not provide conclusive evidence for such a mechanism.

The assumption made in the Theory section that the reaction mechanism is not altered by changes in the structure of the nucleotide substrate is supported by quantitative analysis of data from studies involving $MgUDP^-$ and $MgGDP^-$ as both substrates and inhibitors of the enzymic reaction (Table IV.2). From these results it appears that there is good agreement between the dissociation constants determined independently by the two methods, indicating that the proposed reaction mechanism is valid with these Mg-nucleotide complexes as substrates. Kinetic constants for $MgdADP^-$ and $MgIDP^-$ could not be determined from inhibition studies, with $MgADP^-$ and phosphoarginine as

substrates, because, under the conditions used in such inhibition studies, these nucleotides function to a significant extent as alternative substrates, thus complicating the reaction. $MgCDP^-$ could not be used as a substrate since the initial velocity of the reaction with this nucleotide as a substrate was very low.

James and Morrison (1966a) have carried out quantitative kinetic investigations on the reverse direction of the creatine kinase reaction with various nucleoside diphosphates as substrates and inhibitors. These indicated that with the Mg-complexes of GDP and UDP as substrates the reaction mechanism is rapid equilibrium, random, with two dead-end complexes, as when $MgADP^-$ is the substrate. This was deduced from the good agreement between the kinetic constants determined using these nucleotides as both substrates and inhibitors of the reaction. With $MgCDP^-$ as the substrate, however, there was some discrepancy between the values for the Michaelis constant, K_n, and the inhibition constant, K_{In}, although the dissociation constants were in good agreement.

In order to determine with greater certainty that the reaction mechanism remains unchanged when the different Mg-nucleotides are used as substrates, it is

possible to do product inhibition and isotope exchange studies with these compounds as substrates. Morrison and White (1967) investigated the effects of different nucleotides on the rates of isotope exchange at equilibrium in the reaction catalysed by creatine kinase, using the nucleotides CDP, GDP and UDP in the presence of 5 mM Mg^{2+}. In contrast with the results of the kinetic studies of James and Morrison (1966a) discussed above, these experiments indicated that the rapid equilibrium condition holds when CDP is present. However, in the presence of GDP and UDP there is a larger discrepancy in the rates of isotope exchange occurring between the nucleotide pair and the guanidino pair of reactants. Thus, although the reaction mechanism of creatine kinase is probably rapid equilibrium, random, with two dead-end complexes irrespective of the nature of the nucleotide substrate, a true rapid equilibrium condition may not hold in all cases. Similarly, with this arginine kinase, it is not possible to be certain, on the basis of the evidence obtained, that the mechanism is rapid equilibrium when $MgADP^-$ is replaced by the Mg-complexes of other nucleoside diphosphates, especially in the light of the observations of Gulbinsky and Cleland

(1968) on the difficulties of demonstrating, by kinetic methods, that a random mechanism is truly rapid equilibrium.

From the kinetic constants presented in Table IV.1(b), it appears that the dissociation constant for the interaction of phosphoarginine with the free enzyme, K_{ip}, does not vary more than three-fold with the different Mg-nucleotide complexes present as substrates. This variation may be due to experimental error, since the combination of phosphoarginine with the free enzyme should not be affected by the structure of the nucleotide substrate available. Similarly, the nature of the nucleotide does not seem to have much effect on the dissociation constant for the interaction of phosphoarginine with the enzyme-Mg-nucleotide complex, K_p, since the value of this kinetic constant does not vary by a factor greater than three. This indicates that the structure of the site for phosphoarginine combination is not affected to a large extent by the nature of the Mg-nucleotide complex already present on the nucleotide site.

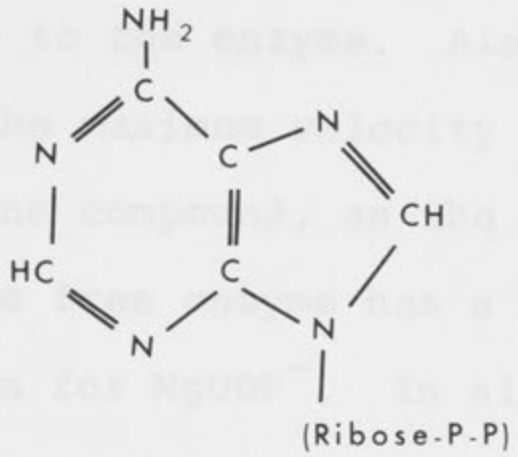
The values for the dissociation constants for the interaction of the various nucleotides with the free enzyme, K_{in}, are given in Table IV.1(a), and these

show variation over approximately two orders of magnitude, indicating that the affinity of the free enzyme for the other Mg-nucleotide complexes is very much lower than for $MgADP^-$. The values of the kinetic constants for the interaction of the nucleotides with the enzyme-phosphoarginine complex, K_n, increase to approximately twenty times that for K_{MgADP^-} when the normal substrate is replaced by $MgUDP^-$ or $MgGDP^-$.

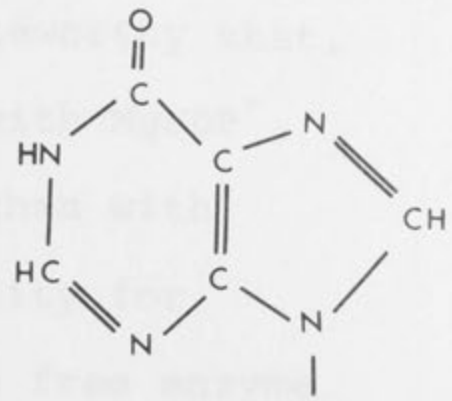
When considering the relationship between the structure of a nucleoside diphosphate substrate analogue and its substrate activity, it is interesting to note the great effect which the substitution of deoxyribose for ribose in $MgADP^-$ has on the maximum velocity of the reaction catalysed by this arginine kinase.

The structures of the nucleotide analogues of ADP used in the form of their Mg-complexes as substrates and inhibitors of the arginine kinase reaction are given on the following page. It is apparent that the Mg-complexes of both purine and pyrimidine nucleoside diphosphates can function as substrates, although $MgCDP^-$ is the least effective substrate of those tested. $MgIDP^-$ interacts more readily with both the free enzyme and the enzyme-phosphoarginine complex than do either $MgUDP^-$ or

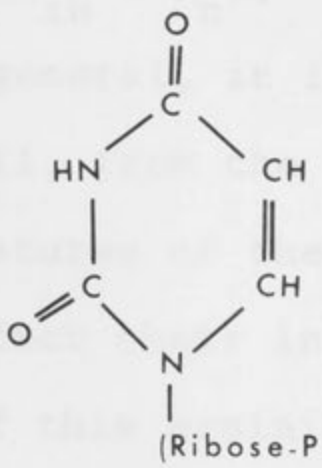
ADP



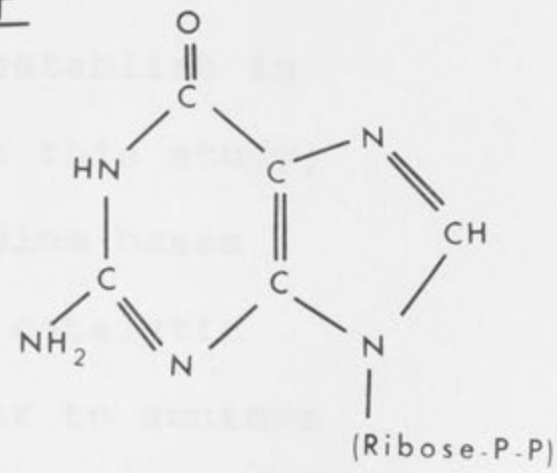
IDP



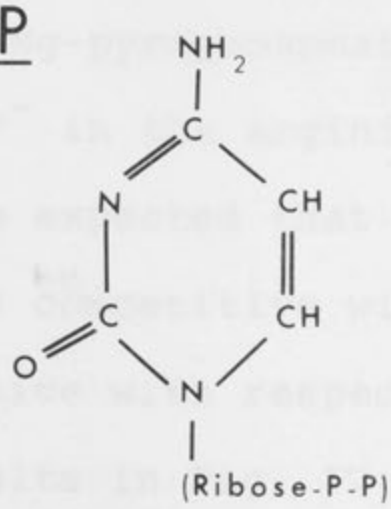
UDP



GDP



CDP



$MgGDP^-$, indicating that the presence of a group in the 2-position may cause hindrance to the binding of the nucleotide to the enzyme. Also, it is noteworthy that, although the maximum velocity is greater with $MgUDP^-$, a pyrimidine compound, as the substrate, than with $MgGDP^-$, the free enzyme has a higher affinity for $MgGDP^-$ than for $MgUDP^-$. In all cases, the free enzyme has a greater affinity for the Mg-complex of the nucleotide than does the enzyme-phosphoarginine complex ($K_{in} < K_n$).

In general, it is not possible to establish in any detail, from the results obtained in this study, those features of the purine and pyrimidine bases which affect their interaction with the catalytic centre of this arginine kinase. In order to achieve this, the effects of a wider range of nucleotide analogues would have to be investigated.

If Mg-pyrophosphate is considered as an analogue of $MgADP^-$ in the arginine kinase reaction, then it would be expected that inhibition by this compound would be competitive with respect to $MgADP^-$ and non-competitive with respect to phosphoarginine. However, the results in Fig. IV.8 show that the inhibition is competitive with respect to both substrates, and that

a replot of slope against inhibitor concentration in each case would be parabolic. From the initial velocity equation for the reaction (equation IV.1),

$$v = \frac{V_2}{\frac{K_{ip}K_q}{PQ} + \frac{K_p}{P} + \frac{K_q}{Q} + 1}$$

it can be shown that the inhibitor, $MgPP_i^{2-}$, (I), can be competitive with respect to both P and Q if it combines only with the free enzyme, E, since if I combines with either EQ or EP as well as with E, then the inhibition is non-competitive with respect to P or Q, respectively. However, since the inhibition is slope parabolic, the inhibitor, I, must react more than once with the free enzyme, and the equation thus becomes :-

$$v = \frac{V_2}{\frac{K_{ip}K_q}{PQ}\left[1 + \frac{I}{K_{I1}} + \frac{I^2}{K_{I2}}\right] + \frac{K_p}{P} + \frac{K_q}{Q} + 1} \qquad (IV.6)$$

where K_{I1} and K_{I2} are kinetic constants associated with the interaction of $MgPP_i^{2-}$ with the free enzyme and enzyme-$MgPP_i$ complex, respectively. This equation (IV.6) can be rearranged in double reciprocal form, with P and Q as the variable substrates to give the equations :-

$$\frac{1}{v} = \frac{K_p}{V_2}\left\{\frac{K_{iq}}{Q}\left[1 + \frac{I}{K_{I1}} + \frac{I^2}{K_{I1}K_{I2}}\right] + 1\right\}\frac{1}{P} + \frac{1}{V_2}\left\{\frac{K_q}{Q} + 1\right\} \quad \text{(IV.7.a)}$$

$$\frac{1}{v} = \frac{K_q}{V_2}\left\{\frac{K_{ip}}{P}\left[1 + \frac{I}{K_{I1}} + \frac{I^2}{K_{I1}K_{I2}}\right] + 1\right\}\frac{1}{Q} + \frac{1}{V_2}\left\{\frac{K_p}{P} + 1\right\} \quad \text{(IV.7.b)}$$

This type of equation provides for parabolic competitive inhibition by Mg-pyrophosphate with respect to both substrates. For this type of inhibition to occur, $MgPP_i^{2-}$ must react twice with the free enzyme, probably at both the nucleotide and guanidino substrate binding sites, and not with either the enzyme-MgADP complex or the enzyme-phosphoarginine complex. Since the dead-end complex enzyme-MgATP-phosphoarginine can be formed (Chapter III) there appear to be binding sites for at least three phosphate groups at the active site of the enzyme. It is conceivable that Mg-pyrophosphate could combine twice with the enzyme to form a dead-end complex enzyme-$(MgPP_i)_2$. However, there is no obvious reason why $MgPP_i^{2-}$ does not combine with either the enzyme-phosphoarginine complex, or the enzyme-MgADP

complex, since presumably both these complexes have at least one phosphate-binding site available for reaction. Steric hindrance may, however, prevent the combination of $MgPP_i^{2-}$ at these sites.

In the case of AMP inhibition of the reverse reaction of arginine kinase, AMP, as an analogue of $MgADP^-$, would be expected to be a competitive inhibitor with respect to this substrate, and a non-competitive inhibitor with respect to phosphoarginine. Similarly, phosphocreatine, as an analogue of phosphoarginine would be expected to give non-competitive inhibition with respect to $MgADP^-$ and competitive inhibition with respect to phosphoarginine. However, the results presented in Figs. IV.9 and IV.10 show that the inhibition by both these compounds is non-competitive with respect to both substrates. These results can be explained if the inhibitor, I, combines with the free enzyme, the enzyme-MgADP complex and the enzyme-phosphoarginine complex. If this occurs, then the initial velocity equation (IV.1) becomes :-

$$v = \frac{V_2}{\frac{K_{ip}K_q}{PQ}\left(1 + \frac{I}{K_{I1}}\right) + \frac{K_p}{P}\left(1 + \frac{I}{K_{I2}}\right) + \frac{K_q}{Q}\left(1 + \frac{I}{K_{I3}}\right) + 1} \qquad \text{(IV.8)}$$

where K_{I1}, K_{I2} and K_{I3} are dissociation constants for the interaction of the inhibitor with the free enzyme, EQ and EP, respectively. This initial velocity equation can be rearranged in double reciprocal form with either phosphoarginine, (P), or $MgADP^-$, (Q), as the variable substrate, to give :-

$$\frac{1}{v} = \frac{K_p}{V_2}\left\{\frac{K_{iq}}{Q}\left(1 + \frac{I}{K_{I1}}\right) + \left(1 + \frac{I}{K_{I2}}\right)\right\}\cdot\frac{1}{P} + \frac{1}{V_2}\left\{1 + \frac{K_q}{Q}\left(1 + \frac{I}{K_{I3}}\right)\right\} \qquad \text{(IV.9.a)}$$

$$\text{and} \quad \frac{1}{v} = \frac{K_q}{V_2}\left\{\frac{K_{ip}}{P}\left(1 + \frac{I}{K_{I1}}\right) + \left(1 + \frac{I}{K_{I3}}\right)\right\}\cdot\frac{1}{Q} + \frac{1}{V_2}\left\{1 + \frac{K_p}{P}\left(1 + \frac{I}{K_{I2}}\right)\right\} \qquad \text{(IV.9.b)}$$

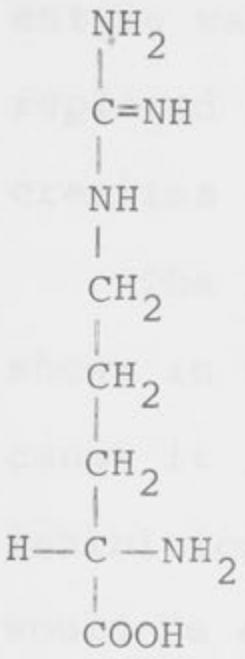
When the equations have this form, both the slopes and intercepts are affected by the concentration of the inhibitor and the inhibition patterns are non-competitive. It is possible that such inhibitions by AMP and phosphocreatine with respect to both $MgADP^-$ and phosphoarginine are due to the presence of a single phosphate group on each of these compounds, so that they can react with the free enzyme and both the enzyme-substrate complexes, since all these have at least one free phosphoryl binding site.

The competitive inhibition by creatine with respect to arginine in the forward direction of the reaction (Fig. IV.11) is consistent with creatine acting only as an analogue of arginine, and therefore reacting only with those forms of enzyme with which arginine reacts. The inhibition by creatine with $MgATP^{2-}$ as the variable substrate was not studied, so it is not possible to determine the dissociation constants for the interaction of creatine with the free enzyme and the enzyme-MgATP complex.

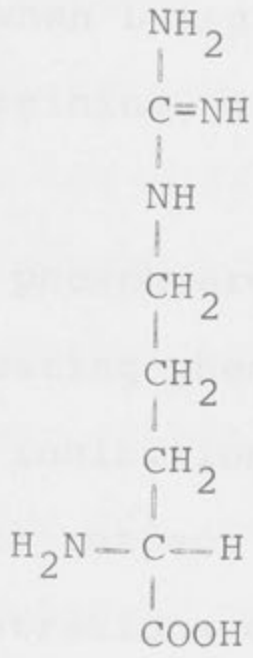
It appears that this arginine kinase is very specific for arginine, since D-arginine, canavanine and creatine, whose structures are shown on the following page, do not function as substrates. The fact that D-arginine functions neither as a substrate nor as an inhibitor of the reaction is of particular interest, because it indicates that the steric conformation of the amino acid is very important in determining binding to the enzyme. However, this is not the only feature of the substrate for which the guanidine binding site is specific, since canavanine, which closely resembles arginine in chain length, could not be shown to have either substrate or inhibitor activity.

Similar results were reported by Virden and Watts

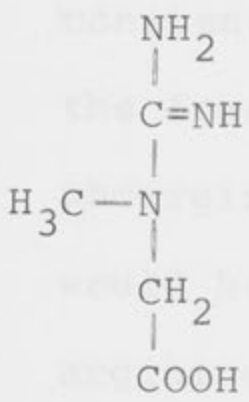
L-arginine



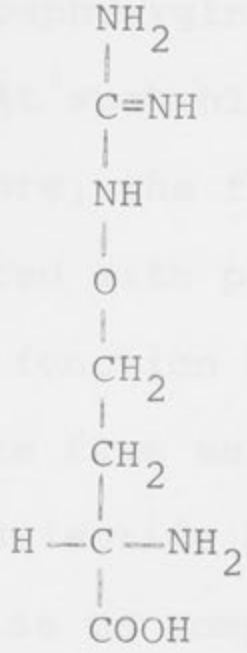
D-arginine



Creatine



L-canavanine



(1964) for the arginine kinase from H. vulgaris. This enzyme was found to be inactive when L-arginine was replaced as the substrate by D-arginine, glycocyamine, creatine or taurocyamine.

The substrate inhibition by phosphoarginine, shown in Fig. IV.12, is an interesting phenomenon because it is not simple substrate inhibition, when the inhibition by phosphoarginine with respect to $MgADP^-$ would be competitive. The concentrations of phosphoarginine required to demonstrate substrate inhibition, ($P \geq 10$ mM), are high relative to the dissociation constant for the interaction of phosphoarginine with the free enzyme ($K_{ip} = 0.26$ mM). At such high phosphoarginine concentrations, therefore, the free enzyme would have been effectively saturated with phosphoarginine, and this substrate could function as an inhibitor by reacting twice with the free enzyme, the second combination involving the nucleotide binding site. This, however, would give rise to competitive inhibition with respect to $MgADP^-$ since $MgADP^-$ also reacts with the free enzyme and the enzyme-phosphoarginine complex. While this type of inhibition may take place, the inhibition must also occur in some other way, and it is conceivable that phosphoarginine could react

twice with the enzyme-MgADP complex, once as a substrate and once as an inhibitor. This would give rise to non-competitive inhibition with respect to $MgADP^-$. However, it appears unlikely that the enzyme-MgADP-phosphoarginine complex would have much affinity for another phosphoarginine molecule because of the number of phosphate groups already present at the active site. Also, the dissociation constant for the interaction of phosphoarginine with the enzyme-MgADP complex is high (K_p = 3.8 mM) relative to that for its interaction with the free enzyme (K_{ip} = 0.26 mM). Phosphoarginine might, however, combine at the nucleotide binding site in such a way that $MgADP^-$ could still react with the enzyme. It seems conceivable, when the structures of the guanidino group of phosphoarginine and the 1, 5 and 6 positions of the adenine moiety are compared, that phosphoarginine could react to a small extent at the adenine-binding site, but in such a way as not to prevent the combination of $MgADP^-$, but to decrease the rate of reaction.

It is also possible, especially in view of the specificity of the guanidine-binding site for L-arginine as opposed to D-arginine, that a molecule of phosphoarginine could be bound to the enzyme in such

a way as to allow the binding of a second phosphoarginine to the phosphoarginine phosphate-binding site. This might then result in the formation of an enzyme-MgADP-(phosphoarginine)$_2$ complex which would not break down to give products.

Another possible explanation for the non-competitive substrate inhibition by phosphoarginine with respect to $MgADP^-$ is that a stable enzyme-MgADP-Mg-phosphoarginine complex, which would not break down to give products, might be formed. Such inhibition could not be overcome by high concentrations of $MgADP^-$, and non-competitive substrate inhibition would therefore be observed. Since the stability constant for Mg-phosphoarginine under the experimental conditions is 100 M^{-1} (O'Sullivan and Perrin, 1964), there is 1 mM Mg-phosphoarginine present when phosphoarginine is at a concentration of 10 mM, and the free Mg^{2+} is 1 mM. If the dissociation constant for the interaction of Mg-phosphoarginine with free enzyme were similar to that for free phosphoarginine (K_{ip} = 0.26 mM), there would be considerable formation of enzyme-Mg-phosphoarginine, with which $MgADP^-$ could react to form a dead-end complex. At 5 mM phosphoarginine the free enzyme has been effectively saturated with this substrate, and higher concentrations of phosphoarginine

would have little effect on the formation of the enzyme-phosphoarginine complex, but concentrations above 10 mM could lead to the formation of an enzyme-Mg-phosphoarginine complex, since significant concentrations of Mg-phosphoarginine would be present. The results given in the next chapter, obtained using pulsed nuclear magnetic resonance, are consistent with the formation of an equivalent enzyme-Mn-phosphoarginine complex.

SUMMARY

1. Initial velocity studies of the reverse direction of the arginine kinase reaction were carried out with $MgdADP^-$, $MgIDP^-$, $MgUDP^-$ and $MgGDP^-$ as substrates.

2. Inhibition of the reverse reaction by $MgUDP^-$, $MgGDP^-$ and $MgCDP^-$ with respect to both $MgADP^-$ and phosphoarginine was studied, and quantitative analysis of the data with $MgUDP^-$ and $MgGDP^-$ as both substrates and inhibitors was consistent with the reaction mechanism of arginine kinase being rapid equilibrium, random, with two dead-end complexes.

3. Dead-end inhibition of the reverse direction of the arginine kinase reaction by $MgPP_i^{2-}$, AMP^{2-} and phosphocreatine was investigated.

4. The effects of the analogues of L-arginine, D-arginine, canavanine and creatine, on the forward direction of the arginine kinase reaction indicated that the enzyme has a high degree of substrate specificity for the guanidino substrate.

5. Substrate inhibition of the reverse reaction of arginine kinase by phosphoarginine was investigated and the inhibition was found to be non-competitive with respect to $MgADP^-$.

CHAPTER V

Magnetic Resonance and Kinetic Studies of the Manganese-Activated Arginine Kinase Reaction

INTRODUCTION

Because the paramagnetic manganous ion can replace Mg^{2+} as the essential metal ion in the arginine kinase reaction, it is possible to utilise magnetic resonance techniques to obtain information about the complexes containing Mn^{2+} and enzyme and/or substrate that may be formed. The results presented in this chapter were obtained using pulsed nuclear magnetic resonance, the so-called "spin-echo" technique. The parameter measured is T_1, the longitudinal relaxation time of water protons, which is greatly affected by the addition of manganous ions, in the first place, and further perturbed when Mn^{2+} is complexed by a ligand.

Nuclear magnetic resonance (NMR) was first used in the study of macromolecules of biological interest by Lumry *et al.* (1961) for the investigation of the structure and denaturation of haem-proteins, and, in its more familiar form, the recording of spectra, is proving to be a very valuable tool in the elucidation of structural features of macromolecules (Bradbury and Crane-Robinson, 1968). The application of pulsed NMR techniques to the study of biological materials was first reported by Eisinger *et al.* (1961) for work

on the binding of various paramagnetic ions to deoxyribonucleic acid. The technique was developed for the study of enzyme-metal-substrate complexes by Cohn and Leigh (1962) who investigated the effects of the paramagnetic ion, Mn^{2+}, on the proton relaxation rates (PRR) of water protons in the presence of ADP and ATP with creatine kinase and bovine serum albumin. These authors also studied the effects of the addition of enolase to a solution containing Mn^{2+} ions, and the results indicated that the metal ion was bound directly to the enzyme, whereas it was not bound to creatine kinase in the absence of nucleotide. Further studies of the interaction of Mn^{2+} with bovine serum albumin were carried out by Mildvan and Cohn (1963) and these showed good agreement between the binding constants obtained independently, either by determination of the free Mn^{2+} using electron paramagnetic resonance, or by measuring the protein-bound Mn^{2+} by determination of the proton relaxation rate. Apart from obtaining quantitatively consistent results for binding constants, the studies reported in this paper demonstrated that the measurement of PRR provided a sensitive probe for following subtle changes in protein conformation such as were induced by low concentrations of the denaturing

agents, urea, guanidinium chloride and decyl sulphate, and could not have been detected by other techniques.

General discussions of the extension of the PRR technique to enzyme complexes have been presented by Cohn (1963, 1967) who has classified enzymes into two types according to the way in which Mn^{2+} interacts with the enzyme. Thus, for enzymes of Type I, such as creatine kinase and muscle 3-phosphoglycerate kinase, the metal ion is bound to the enzyme through the nucleotide substrate, while for enzymes of Type II, such as yeast enolase and muscle pyruvate kinase, the metal ion is bound directly to the enzyme and, in the latter case, probably functions as a bridge between the enzyme and the nucleotide substrate (Mildvan et al., 1967). A list of the enzymes which have been shown to belong to each of these categories has been compiled by Cohn (1967).

Detailed studies, using proton relaxation rate measurements, have been carried out on the complexes formed in the creatine kinase reaction (O'Sullivan and Cohn, 1966a, 1966b) and in the pyruvate kinase reaction (Mildvan and Cohn, 1965; Mildvan et al., 1967), with Mn^{2+} as the activating ion. Also, PRR studies have been used by O'Sullivan et al. (1969) in the investig-

ation of the Mn-activated reaction of arginine kinases from H. vulgaris and H. americanus with ADP, dADP, ATP and dATP as substrates. It was found that both these enzymes are of Type I. However, the reaction mechanisms of these enzymes have not been established by detailed kinetic studies, although the results of initial velocity studies by Virden et al. (1965) on the enzyme from H. vulgaris indicated a sequential mechanism. The results of PRR studies were also consistent with such a mechanism.

Proton relaxation rate studies with the arginine kinase from the West Australian crayfish, P. longipes, were undertaken and the results compared with those obtained from the kinetic investigations of reaction mechanism of the enzyme, and also with the results previously obtained using the enzymes from H. vulgaris and H. americanus.

THEORY

The manganous ion, because of its five unpaired electrons, is paramagnetic and the interaction of this ion with its immediate environment can be assessed by the proton relaxation rate of the water protons in its first hydration sphere. In pure water the principal mechanism of proton relaxation is due to the magnetic dipolar interaction between protons. However, in the presence of the paramagnetic ion, Mn^{2+}, the proton relaxation time, T_1, is dominated by the much greater proton-electron interaction between water protons and the unpaired electrons of the Mn^{2+} ion. Thus, Mn^{2+} has the effect of increasing the proton relaxation rate, $1/T_1$. For example, it is found that a 1 M solution of Mn^{2+} has a proton relaxation rate approximately 10,000 times greater than that of pure water. The exchange of protons as water molecules between the bulk of an aqueous solution and the first hydration sphere of the Mn^{2+} ion is extremely rapid, and the effect observed is the average of the relaxation rates of protons in both environments. However, the effect due to the ions in the bulk of the solution can be subtracted from the overall effect, so that the paramagnetic contribution, T_{1p}, to the observed relaxation time, T_1, can be determined.

Since the dominant contribution to the effect is due to the interaction of Mn^{2+} with the protons in its first hydration sphere, the magnitude of the effect is very sensitive to the immediate environment of the ion. The replacement of water in the manganous hydration sphere by other ligands, such as EDTA, decreases the effectiveness of Mn^{2+} in increasing the proton relaxation rate, because fewer water molecules are then in close proximity to the ion. However, some compounds, such as deoxyribonucleic acids and some proteins, have the opposite effect, and cause greatly increased proton relaxation rates when added to a Mn^{2+} solution. This effect is known as enhancement. If the Mn^{2+} ion were buried within the macromolecule, there could be no exchange of the water protons in the first hydration sphere with those in the bulk of the solution, and, thus, the effect would be equivalent to removing the ion from solution, or adding a ligand such as EDTA, causing a decrease in the proton relaxation rate. Enhancement, therefore, can occur only when the Mn^{2+} ion is on the outside of the macromolecule, and has been explained by Eisinger et al. (1962) as being due to the hindrance of the rotational motion of the water molecules remaining in

the complex. The increase in proton relaxation rate, or enhancement, is measured by the enhancement factor, ε, which is defined as the ratio of relaxation rates of water protons in the presence and absence of the complexing agent :-

$$\varepsilon^* = \frac{R_1^*}{R_1} \qquad (V.1)$$

where R_1 is the proton relaxation rate due to Mn^{2+} and * indicates the presence of the complexing agent. Since R_1 is the reciprocal of the proton relaxation time, T_1,

$$\varepsilon^* = \frac{R_1^*}{R_1} = \frac{\frac{1}{T_1^*} - \frac{1}{T_1^*(o)}}{\frac{1}{T_1} - \frac{1}{T_{1(o)}}} \qquad (V.2)$$

where T_1 is the observed relaxation time and $T_{1(o)}$ is the relaxation time in the absence of Mn^{2+}.

A further discussion of the parameters that determine the enhancement of enzyme complexes containing Mn^{2+} has been given by Cohn (1967).

EXPERIMENTAL PROCEDURE

Materials

Analar $MnCl_2$ was obtained from British Drug Houses Ltd., treated with dithizone and standardised as described by Morrison et al. (1961). All other chemicals used were as described in Chapters I and II. Arginine kinase, prepared by the method described in Chapter I, was dialysed exhaustively against 0.01 M N-ethylmorpholine-HCl buffer (pH 8.0), to remove any EDTA, and concentrated to 32.4 mg/ml by pressure dialysis. The protein concentration was determined by the Biuret method (Chapter I).

Methods

Determination of the PRR

Proton relaxation times of solutions were measured with a pulsed apparatus of the type described by Carr and Purcell (1954). The protons were held in a fixed alignment by a strong magnetic field and a pulse of radiofrequency energy was passed at right angles to the magnetic field. The time taken for the protons to return to their original alignment after this disturbance was measured by a second sampling pulse. The amplitude of the signal following the sampling pulse is proportional to the magnitude of the magnetisation in the sample. The time interval between

the two pulses is varied until a null signal is obtained. This time interval, τ_{null}, is related to the relaxation time, T_1, by $\tau_{null}/T_1 = \ln 2$.

Measurements of the proton relaxation rates were made using a 90-180° pulsed nuclear magnetic resonance spectrometer, operating at 30 megacycles per sec, which was assembled by Mr. K. Marsden of the Physics Department, University of New South Wales. The position of the null was determined visually on a Textronix, Inc. Type 535 Oscilloscope and readings were taken from an Elron Minicounter. The observed enhancement of the PRR, ε^*, in systems containing manganese and a complexing agent has been defined (Eisinger et al., 1961; Cohn, 1963) as the ratio of the paramagnetic contribution to the relaxation rate, $1/T^*_{1p}$, for the solution containing the complexing agent to $1/T_{1p}$ for the same solution in the absence of the complexing agent :-

$$\varepsilon^* = \frac{\frac{1}{T^*_{1p}}}{\frac{1}{T_{1p}}} \qquad (V.3)$$

The observed relaxation rate, $1/T^*_1$, is the sum of contributions from the paramagnetic species, the complexing agent and the water; to obtain ε^* from

the observed relaxation rates it is assumed that each contribution is independent so that

$$\varepsilon^* = \frac{\frac{1}{T^*_{1p}}}{\frac{1}{T_{1p}}} = \frac{\frac{1}{T^*_1} - \frac{1}{T^*_{1(o)}}}{\frac{1}{T_1} - \frac{1}{T_{1(o)}}} \qquad (V.4)$$

where T_1 and $T_{1(o)}$ are the observed longitudinal relaxation times of water in the buffer solutions, with and without manganese, respectively, and * indicates the presence of a complexing agent. In a solution, $1/T^*_{1p}$ and thus ε^*, will be a weighted average of each paramagnetic species present (c.f. Equation 2 of Mildvan and Cohn, 1963),

$$\varepsilon^* = \frac{Mn_f}{Mn_t} \cdot \varepsilon_f + \frac{Mn_b}{Mn_t} \cdot \varepsilon_b \qquad (V.5)$$

Determination of enhancement for enzyme-manganese-substrate complexes (ε_t)

The analysis given below follows that of O'Sullivan and Cohn (1966a).

For a solution containing enzyme, metal and substrate, the observed enhancement, ε^*, may contain contributions from four terms, viz :-

$$\varepsilon^* = \frac{[M]}{[M]_T} \cdot \varepsilon_f + \frac{[MS]}{[M]_T} \cdot \varepsilon_a + \frac{[EM]}{[M]_T} \cdot \varepsilon_b + \frac{[EMS]}{[M]_T} \cdot \varepsilon_t \qquad (V.6)$$

In this equation M represents the manganese: S, the substrate; E, the enzyme; MS, the manganese-substrate complex; EM, the enzyme-manganese complex, and EMS the enzyme-manganese-substrate complex. ε_a, ε_b, ε_f and ε_b are the enhancements due to the MS complex, the EM complex, the free metal and the EMS complex, respectively. For an enzyme of Type I, such as arginine kinase, the enhancement due to the EM complex, ε_b, is small. To facilitate analysis, experiments are carried out at relatively high values of S, so that the term in ε_b becomes negligible. Under these circumstances, equation V.6 is reduced to :-

$$\varepsilon^* = \frac{[M]}{[M]_T}.\varepsilon_f + \frac{[MS]}{[M]_T}.\varepsilon_a + \frac{[EMS]}{[M]_T}.\varepsilon_t \qquad (V.7)$$

In determining ε_t, the enhancement due to the EMS complex, the following relationships are employed :-

$$[M]_T = [M] + [MS] + [EMS] \qquad (V.8)$$

$$K_1 = \frac{[M][S]}{[MS]} \qquad (V.9)$$

$$K_2 = \frac{[E][MS]}{[EMS]} \qquad (V.10)$$

and

$$K_s = \frac{[E][S]}{[ES]} \qquad (V.11)$$

Equation V.7 can then be expressed as :-

$$\varepsilon^* = \frac{\frac{K_1K_2}{[E][S]} + \frac{K_2}{[E]}\cdot\varepsilon_a + \varepsilon_t}{\frac{K_1K_2}{[E][S]} + \frac{K_2}{[E]} + 1} \qquad (V.12)$$

A plot of ε^* against S for constant E and constant M first increases and then decreases with increasing S, as S competes with MS for the enzyme. The ascending limb of such a curve, when MS is low, is used to determine ε^*_c, the enhancement at finite enzyme concentration and infinite S. This is done using a double reciprocal plot of ε^* against S, which approximates to a straight line, and can thus be extrapolated to determine ε^*_c. If such curves are obtained with several enzyme concentrations, then a replot of $1/\varepsilon^*_c$ against $1/[E]_T$, extrapolated to infinite enzyme concentration, gives the value of ε_t. In the limit, where $[S]_T \to \infty$, the first term in both the numerator and denominator of equation V.12 becomes insignificant and

$$\varepsilon^*_c = \frac{\frac{K_2}{[E]}\cdot\varepsilon_a + \varepsilon_t}{\frac{K_2}{[E]} + 1} \qquad (V.13)$$

Thus, at high values of E, $[E] \to [E]_T$, and the first term in the numerator of equation V.13 becomes small,

so that :-

$$\varepsilon_c^* \approx \frac{\varepsilon_t}{\frac{K_2}{[E]_T} + 1} \qquad (V.14)$$

In reciprocal form this becomes :-

$$\frac{1}{\varepsilon_c^*} \approx \frac{K_2}{\varepsilon_t} \cdot \frac{1}{[E]_T} + \frac{1}{\varepsilon_t} \qquad (V.15)$$

Then a plot of $1/\varepsilon_c^*$ against $1/[E]_T$, when extrapolated to $[E]_T = \infty$, gives the value of $1/\varepsilon_t$ from the intercept on the $1/\varepsilon_c^*$ axis.

Determination of the dissociation constant, K_2

When ε_t has been determined, K_2 can be estimated from the double reciprocal plot of ε_c^* against $[E]_T$ (c.f. O'Sullivan and Noda, 1968). The dissociation constant, K_2, is obtained from the half maximal value of ε_c^*, when half the metal-nucleotide is bound to the enzyme and half is free, so that [MS] = [EMS].

Thus, $K_2 = \frac{[E][MS]}{[EMS]} = [E]$

now, $[E]_T = [E] + [EMS]$

so that, $[E] = [E]_T - [EMS]$

But $[M]_T = [MS] + [EMS]$, so that $[EMS] = \frac{[M]_T}{2}$

and $K_2 = [E]_T - \frac{[M]_T}{2}$

Since $[E]_T$ has been estimated from the double reciprocal plot of ε_c^* against $[E]_T$ and $[M]_T$ is known from the concentration of metal ion originally present, K_2 can be calculated.

Method of "titration"

Experiments were carried out in 0.05 M N-ethylmorpholine-HCl buffer, pH 8.0, with a total concentration of $MnCl_2$ of 0.12 mM and various concentrations of nucleotides and arginine kinase. Titrations were usually carried out in the following manner : two solutions were made up to contain the same concentrations of manganese chloride, enzyme and buffer, in a total volume of 0.1 ml. One of the tubes, (A), contained no nucleotide, and the other, (A'), contained ADP or ATP at a two- or three-fold molar excess over the $MnCl_2$ concentration. Higher concentrations of nucleotides were used when titrations were being carried out with higher enzyme concentrations. The proton relaxation times of the tubes were recorded, and 5, 10, or 20 µl volumes were transferred from A' to A, and the proton relaxation time determined after each addition.

RESULTS

The complexes of Mn^{2+} with nucleotides to form $MnADP^-$ and $MnATP^{2-}$ were taken to have enhancements (ε_a) of 1.6 and 1.7, respectively. The stability constant for the formation of $MnADP^-$ was assumed to be $2.5 \times 10^4\ M^{-1}$, and that for $MnATP^{2-}$, $1 \times 10^5\ M^{-1}$, under the experimental conditions (O'Sullivan and Cohn, 1966a, 1966b).

Complexes of Mn^{2+} with arginine kinase

In the presence of 0.12 mM $MnCl_2$ and arginine kinase over the concentration range 0.121 mM to 0.35 mM a very slight increase in enhancement was observed, and a double reciprocal plot of ε^* against [E] (Mildvan and Cohn, 1965) yielded a value of $\varepsilon_b = 2.86$. The enhancement is decreased in the presence of KCl, indicating that the direct binding of Mn^{2+} to the enzyme is probably non-specific. This is illustrated by the fact that with $MnCl_2 = 1.2 \times 10^{-4}$ M and arginine kinase at a concentration of 2.63×10^{-4} M, $\varepsilon = 2.22$, and in the presence of 0.25 M KCl, $\varepsilon = 1.68$ with the same concentrations of enzyme and $MnCl_2$.

Complexes of Mn^{2+} with nucleotides and arginine kinase

A large increase in enhancement was observed when Mn^{2+} was added to a solution containing arginine kinase

and a nucleotide, either ADP^{3-} or ATP^{4-}, indicating the formation of the complexes, enzyme-MnADP and enzyme-MnATP. It seems, therefore, that the metal ion is bound to the enzyme only through the formation of a metal-nucleotide complex, as has been shown for creatine kinase (Cohn and Leigh, 1962; O'Sullivan and Cohn, 1966a), adenylate kinase (O'Sullivan and Noda, 1968) and the arginine kinases from H. vulgaris and H. americanus (O'Sullivan et al., 1969). With increasing ADP concentration the values of ε for any given enzyme and Mn^{2+} concentration increased to a plateau value and then decreased slightly. Although the decrease in enhancement was less than that observed with equivalent concentrations of creatine kinase, the results are qualitatively similar, suggesting that, as with creatine kinase (Cohn, 1963), the decrease in enhancement was due to competition between free ADP^{3-} and $MnADP^{-}$ for the nucleotide site on the enzyme.

Double reciprocal plots of enhancement, (ε^*), against ADP concentration at three different concentrations of arginine kinase, 0.121, 0.175 and 0.263 mM, were obtained using the ascending limb of the titration curves (Fig. V.1). The extrapolated lines gave values

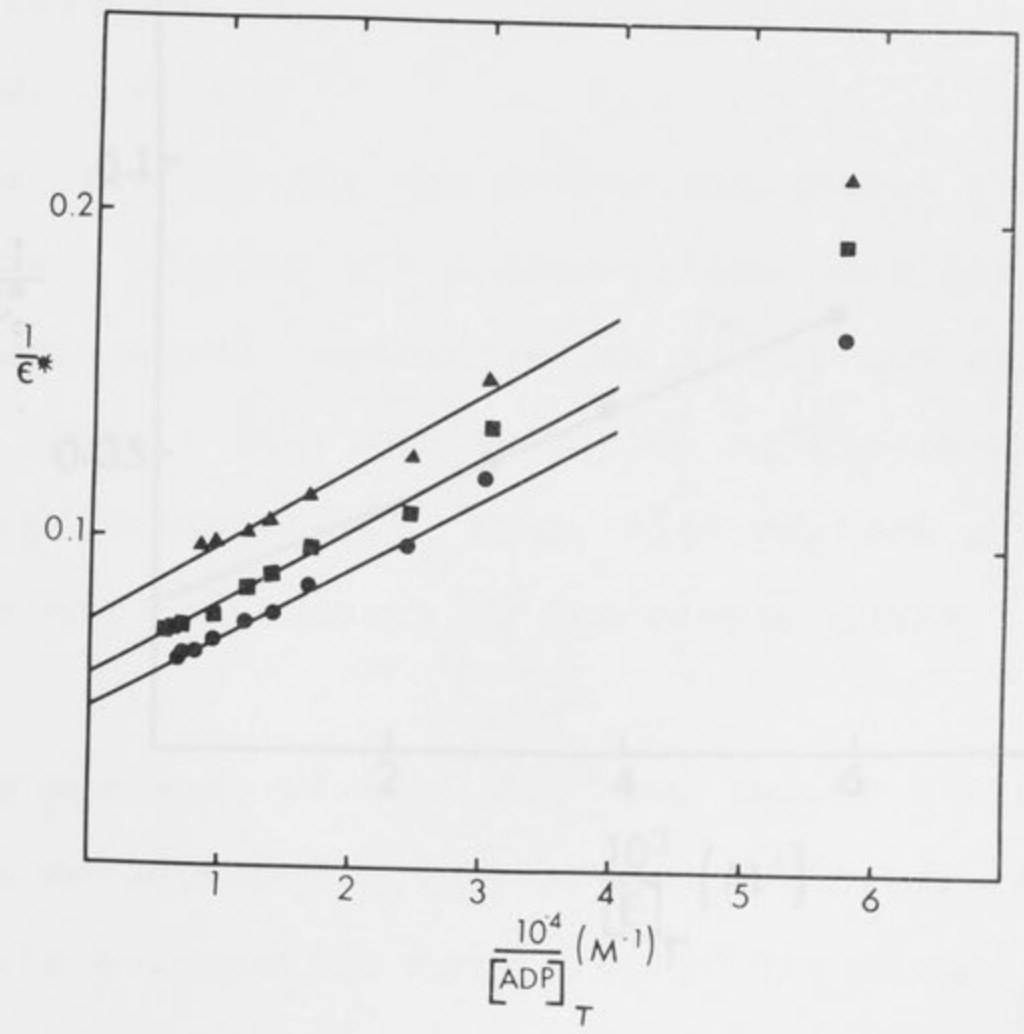


Fig. V.1. Titration of 0.12 mM $MnCl_2$ with ADP at three different concentrations of arginine kinase. The concentrations of arginine kinase were : ●-●-●, 0.121 mM; -■-■-, 0.175 mM; -▲-▲-, 0.263 mM. The titrations were carried out at approximately 20° in 0.05 M N-ethylmorpholine-HCl buffer, pH 8.0, in a total volume of 0.1 ml.

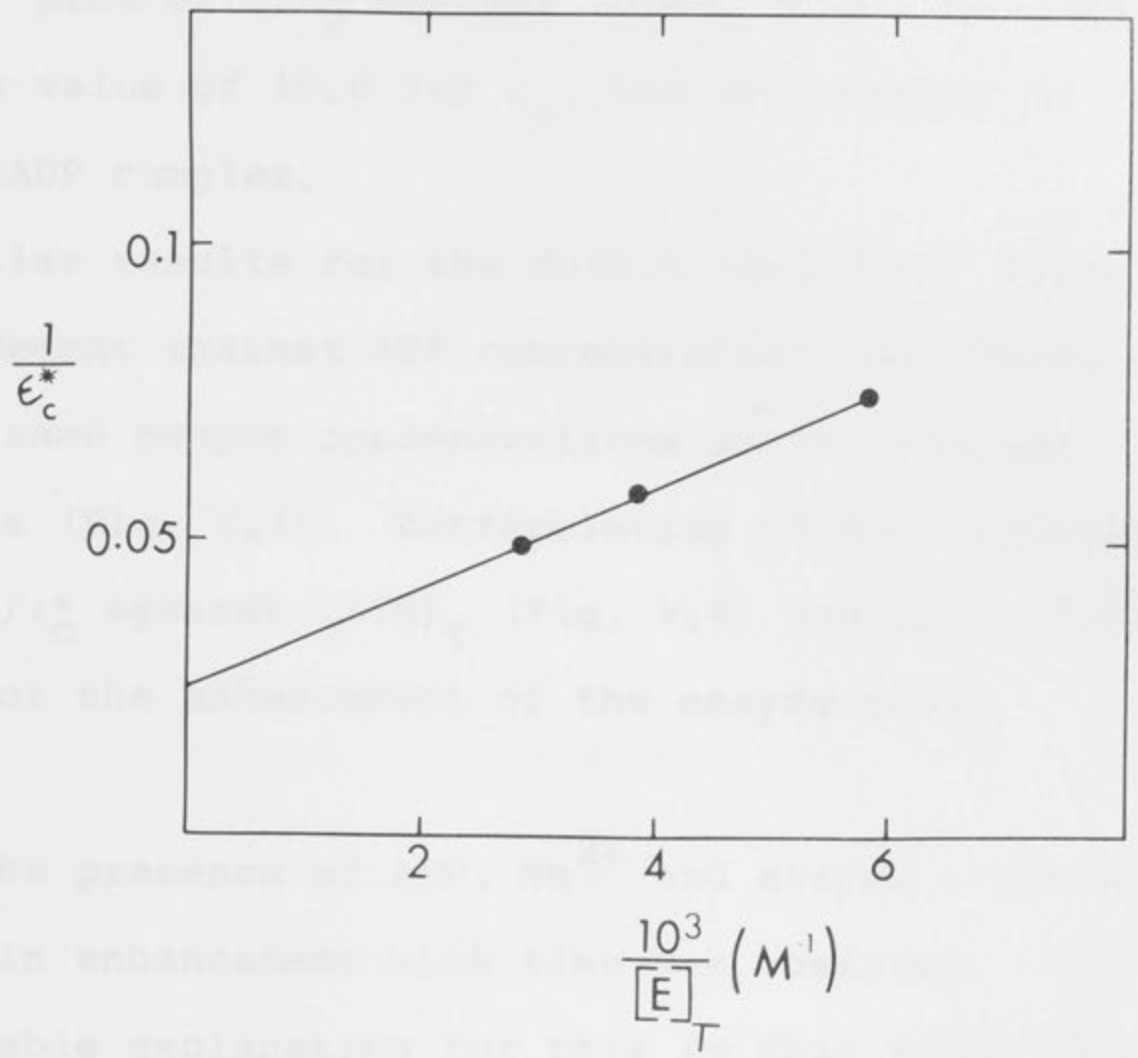


Fig. V.2. Double reciprocal plot of ε_c^* values, obtained from Fig. V.1, against arginine kinase concentration. The line extrapolates to give a value of ε_t equal to 40.8 at infinite enzyme concentration.

for ε_c^*, which is the enhancement at infinite substrate concentration and finite enzyme concentrations. A secondary plot of $1/\varepsilon_c^*$ against $1/[E]_T$ (Fig. V.2) then yielded a value of 40.8 for ε_t, the enhancement of the enzyme-MnADP complex.

Similar results for the double reciprocal plots of enhancement against ATP concentration were obtained with the same enzyme concentrations as for the ADP titrations (Fig. V.3). Extrapolation of the secondary plot of $1/\varepsilon_c^*$ against $1/[E]_T$ (Fig. V.4) yielded a value of 14.9 for the enhancement of the enzyme-MnATP complex.

In the presence of ATP, Mn^{2+} and enzyme a slight increase in enhancement with time was observed. The most probable explanation for this is that $MnADP^-$ was being formed and giving rise to the enzyme-MnADP complex which has a higher enhancement than the enzyme-MnATP complex. Thus, it appears that the arginine kinase has a weak ATPase activity similar to that reported for creatine kinase (Cohn and Leigh, 1962; Sasa and Noda, 1964; O'Sullivan and Cohn, 1966a) and for hexokinase (Kaji and Colowick, 1965).

A considerable increase in enhancement was observed with dADP (3.5×10^{-4} M) under conditions

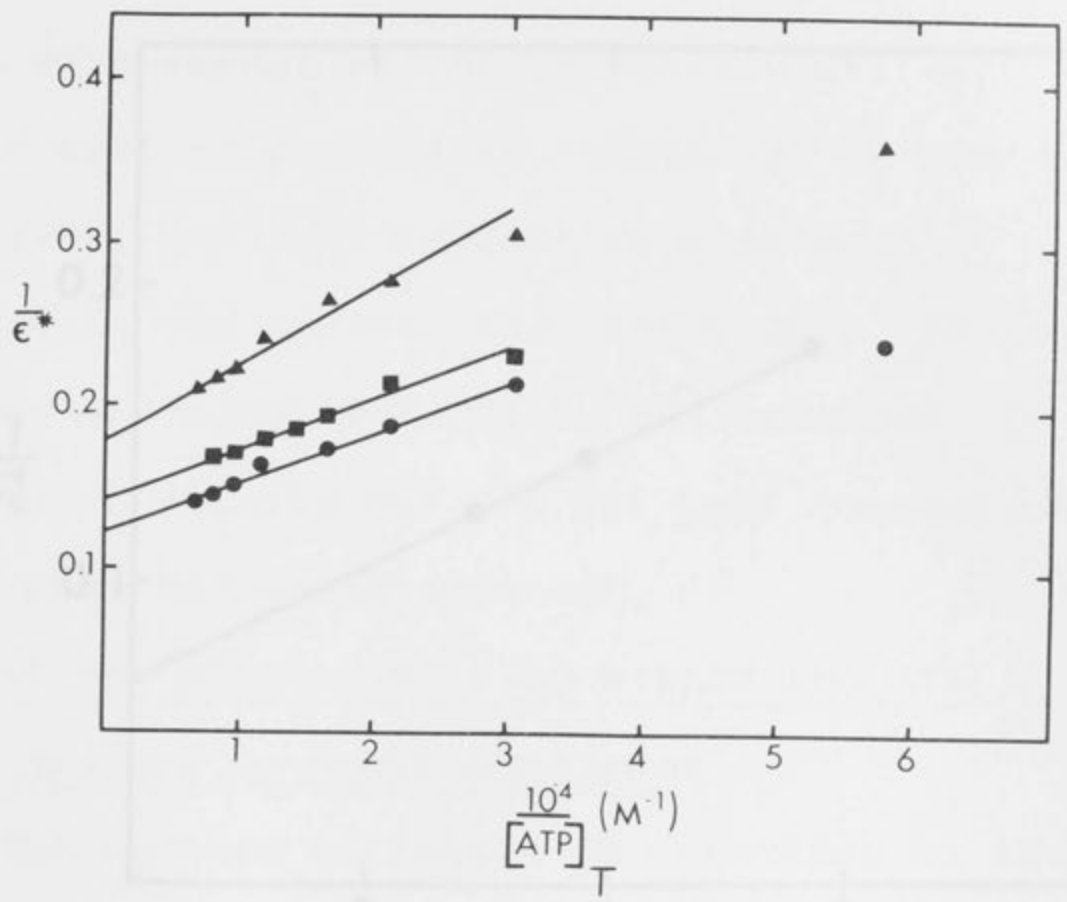


Fig. V.3. Titration of 0.12 m$\underline{M}$ $MnCl_2$ with ATP at three different concentrations of arginine kinase. The concentrations of arginine kinase were : -●-●-, 0.121 mM; -■-■-, 0.175 mM; -▲-▲-, 0.263 mM. The titrations were carried out at approximately 20° in 0.05 M N-ethylmorpholine-HCl buffer, pH 8.0, in a total volume of 0.1 ml.

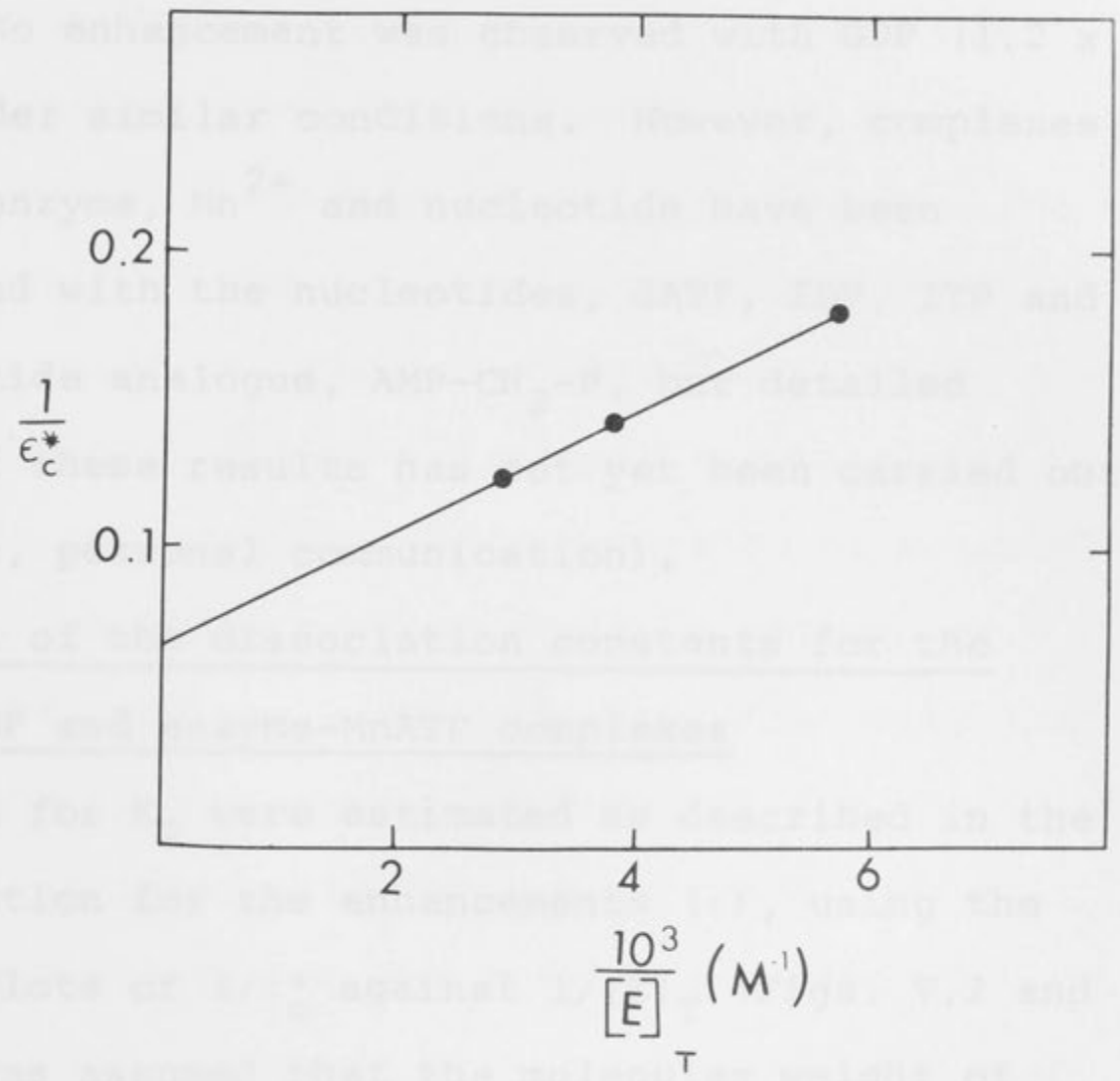


Fig. V.4. Double reciprocal plot of ε_c^* values, obtained from Fig. V.3, against arginine kinase concentration. The line extrapolates to give a value of ε_t equal to 14.9 at infinite enzyme concentration.

where $MnCl_2$ = 4.0 x 10^{-4} M and the concentration of arginine kinase was 2.63 x 10^{-4} M. The value of ε_c^* was 7.1, as compared with 13.7 for the enzyme-MnADP complex. No enhancement was observed with GDP (1.2 x 10^{-3} M) under similar conditions. However, complexes involving enzyme, Mn^{2+} and nucleotide have been demonstrated with the nucleotides, dATP, IDP, ITP and the nucleotide analogue, AMP-CH_2-P, but detailed analysis of these results has not yet been carried out (O'Sullivan, personal communication).

Calculation of the dissociation constants for the enzyme-MnADP and enzyme-MnATP complexes

Values for K_2 were estimated as described in the Methods section for the enhancements (ε), using the secondary plots of $1/\varepsilon_c^*$ against $1/[E]_T$ (Figs. V.2 and V.4). It was assumed that the molecular weight of the enzyme was 40,000 (Chapter VI).

For K_2 for the enzyme-MnADP complex, assuming [MS] = [EMS], $\varepsilon_c^* = \frac{\varepsilon_t + \varepsilon_a}{2} = \frac{40.8 + 1.6}{2}$ = 21.2 and $1/\varepsilon_c^*$ = 0.047. From Fig. V.2, at $1/\varepsilon_c^*$ = 0.047, $[E]_T$ = 0.38 x 10^{-3} M and $K_2 = [E]_T - [M]_T/2$ = (0.38 - 0.06) x 10^{-3} M = 0.32 mM.

For K_2 for the enzyme-MnATP complex, similarly, ε_c^* = 8.31 and $1/\varepsilon_c^*$ = 0.120. Thus, from Fig. V.4,

$[E]_T = 0.364 \times 10^{-3}$ M, so that $K_2 = 0.30$ mM.

Formation of enzyme-MnADP-guanidine complexes

The formation of a dead-end complex with L-arginine and the enzyme-MnADP complex was deduced from the decrease in enhancement when arginine (5×10^{-3} M) was added to a solution containing $MnCl_2$ (1.2×10^{-4} M), ADP (1.2×10^{-4} M) and arginine kinase (2.63×10^{-4} M). This effect could be titrated (Table V.1). From this "titration", which showed saturation behaviour, a dissociation constant for the interaction of L-arginine with the enzyme-MnADP complex could be determined. The value obtained was 1 mM. It should be noted that this is not a true dissociation constant for the interaction of L-arginine with the enzyme-MnADP complex, since the enzyme was not completely saturated with $MnADP^-$. However, it is in reasonable agreement with the value of 0.55 mM for K_{Iq} reported in Chapter III (Table III.4) for the interaction of L-arginine with the enzyme-MgADP complex.

Both D-arginine, which does not combine with the enzyme to a significant extent (Chapter IV, p.128), and creatine, which is a competitive inhibitor with respect to arginine (Chapter IV, Fig. IV.11) were used as controls for the demonstration of the formation

Table V.1.

Variation of PRR enhancement of E-MnADP complex in presence of guanidines.

Various concentrations of L-arginine were added to solutions containing 0.12 mM $MnCl_2$ and 0.12 mM ADP. D-Arginine and creatine were also added to solutions with the same concentrations of $MnCl_2$ and ADP. In all cases the arginine kinase concentration was 2.63×10^{-4} M.

Guanidine	Concentration (mM)	ε*
None	-	8.57
L-arginine	0.83	6.19
	1.43	5.45
	1.88	5.14
	2.22	4.73
	5.0	4.21
D-arginine	6.0	9.29
Creatine	4.0	10.73

of an enzyme-MnADP-L-arginine complex. Creatine (4×10^{-3} M) and D-arginine (6×10^{-3} M) both caused slight increases in enhancement when added to solutions containing $MnCl_2$, ADP and enzyme (Table V.1), indicating that the effect observed with L-arginine was not a non-specific effect, but that an enzyme-MnADP-L-arginine complex was in fact formed.

An interesting feature of this study was that temperature did not affect the proton relaxation time, T_1, of the enzyme-MnADP complex, whereas a decrease in temperature from approximately 20° to approximately 0° had a marked effect in decreasing the T_1 of the enzyme-MnADP-L-arginine complex. Decreasing the temperature at which the measurements were carried out did not, however, significantly affect the T_1 of the enzyme-MnADP complex in the presence of either D-arginine or creatine. This supports the view that enzyme-MnADP-L-arginine is formed by specific binding of L-arginine to the enzyme-MnADP complex.

Evidence for binding of phosphoarginine to arginine kinase

An increase in enhancement was observed when phosphoarginine (5×10^{-3} M) was added to a solution containing arginine kinase (2.63×10^{-4} M) and $MnCl_2$ (1.2×10^{-4} M), indicating that a complex involving

Mn^{2+}, enzyme and phosphoarginine was formed. Moderate binding of Mn^{2+} to phosphoarginine would be expected since the stability constant for the interaction of Mg^{2+} with phosphoarginine under the conditions of the experiment is 100 M^{-1} (O'Sullivan and Perrin, 1964). Assuming a similar stability constant for the interaction of Mn^{2+} with phosphoarginine, and a dissociation constant of 0.14 $\pm$ 0.06 mM for the interaction of phosphoarginine with the free enzyme (Table V.2), some increase in enhancement might be expected due to the binding of Mn^{2+} to phosphoarginine, and phosphoarginine to arginine kinase.

Kinetic studies of the reverse reaction of arginine kinase with $MnADP^-$ as a substrate

Initial velocity studies were carried out as described in Chapter II with $MnADP^-$ as a substrate of the reverse reaction. The initial velocity pattern obtained is shown in Fig. V.5, and, by computer fitting of the data to the SEQUEN programme (Preface, equation 5), the value of K_{iq}, (K_2 for $MnADP^-$) was determined and is shown in Table V.2. These values are calculated on the assumption that the mechanism of the reaction is rapid equilibrium, random, with two dead-end complexes, as when Mg^{2+} is the activating

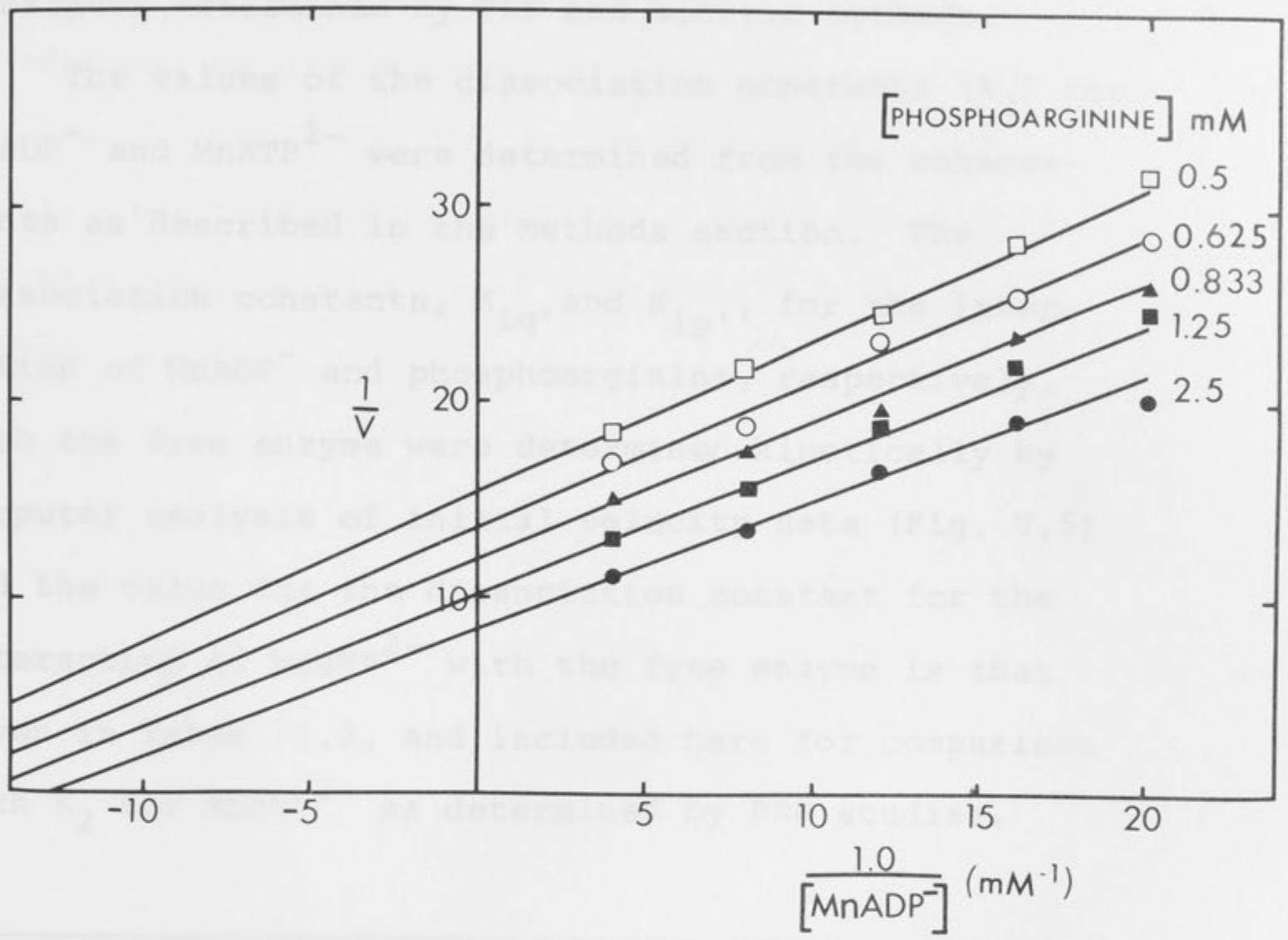


Fig. V.5. Initial velocity pattern obtained with $MnADP^-$ as the variable substrate and phosphoarginine at a number of fixed concentrations. Velocities are expressed as μmoles per min per μg of arginine kinase.

Table V.2.

Summary of the enhancements, and of the dissociation constants determined by PRR and kinetic methods.

The values of the dissociation constants (K_2) for $MnADP^-$ and $MnATP^{2-}$ were determined from the enhancements as described in the Methods section. The dissociation constants, $K_{iq'}$ and $K_{ip'}$, for the interaction of $MnADP^-$ and phosphoarginine, respectively, with the free enzyme were determined kinetically by computer analysis of initial velocity data (Fig. V.5) and the value for the dissociation constant for the interaction of $MgATP^{2-}$ with the free enzyme is that given in Table II.2, and included here for comparison with K_2 for $MnATP^{2-}$ as determined by PRR studies.

Complex	ε_t	K_2 (mM)	K_i (mM)
Enzyme-MnADP	40.8	0.32	0.02 $\pm$ 0.009
Enzyme-MnATP	14.9	0.30	-
Enzyme-MgATP	-	-	0.34 $\pm$ 0.04
Enzyme-phospho-arginine	-	-	0.14 $\pm$ 0.06

ion. Initial velocity experiments in the forward direction of the reaction, with $MnATP^{2-}$ as a substrate were attempted, but, over the ranges of substrate concentrations used, there was little if any change in slope of the lines in double reciprocal plots of initial velocity against substrate concentration, and therefore values of K_{ia}, (K_2 for $MnATP^{2-}$) could not be determined.

DISCUSSION

The results of the PRR studies on the arginine kinase from P. longipes show that the enzyme is of Type I (Cohn, 1963) and is, in this respect, similar to creatine kinase and the arginine kinases from H. vulgaris and H. americanus (O'Sullivan et al., 1969). As was found with the arginine kinase from H. vulgaris, ε_t for the enzyme-MnADP complex was considerably higher than that for the enzyme-MnATP complex.

The dissociation constants for the interaction of $MnADP^-$ and $MnATP^{2-}$ with the free enzyme were calculated to be 0.32 mM and 0.30 mM, respectively. Initial velocity studies of the reverse direction of the Mn-activated arginine kinase reaction yielded a value of 0.02 $\pm$ 0.009 mM for the dissociation constant for the interaction of $MnADP^-$ with the free enzyme (Table V.2). Although no value was obtained for the dissociation constant for the interaction of $MnATP^{2-}$ with the free enzyme, it is probably similar to that determined with $MgATP^{2-}$ (Chapter II, Table II.2), which is 0.34 mM. Thus there is good agreement between the dissociation constants for the interaction of the metal complexes of ATP^{4-} with the free enzyme. For $MnADP^-$, on the other hand, there is a considerable

discrepancy between the values obtained for the dissociation constant for the interaction of the complex with arginine kinase as determined by kinetic and PRR methods. The reason for this discrepancy is not clear. However, one factor could be the very large differences in enzyme concentrations in the two types of experiment. For PRR experiments, the enzyme is present at concentrations of the order of 3 to 10 mg/ml, while for kinetic studies the enzyme concentration does not exceed 0.3 µg/ml. Also, it may be noted that the graphical analysis used here can sometimes be subject to considerable error, since a plot of $1/\varepsilon^*$ against $1/S$ is, in fact, a curve, although it approximates to a straight line under certain conditions. Therefore, if points outside this optimal region of the curve are selected and used in the extrapolation, then erroneous values for ε_c^*, and thus for ε_t and K_2 may be obtained (O'Sullivan, personal communication). A computer programme for the calculation of ε_t and K_2 from the experimentally determined values of ε^* is at present being developed (G. Reed and M. Cohn, personal communication to W.J. O'Sullivan) and this should give more reliable values for the constants.

There is good evidence for the formation of a dead-end enzyme-MnADP-L-arginine complex, since the addition of L-arginine to the enzyme-MnADP complex caused a considerable decrease in enhancement (Table V.1), and this was significantly affected by changes in temperature. D-Arginine and creatine, however, had only small effects on the enhancement of the enzyme-MnADP complex. These effects were in the direction of increased enhancement and the proton relaxation times were not altered by temperature changes. Similar results were reported by O'Sullivan et al. (1969) for the H. vulgaris arginine kinase. Also, the effects of temperature are similar to those reported by O'Sullivan and Cohn (1968), who found that for creatine kinase the PRR of the enzyme-MnADP complex was independent of temperature, but the addition of creatine to this complex gave rise to a complex whose PRR had a positive temperature coefficient. In this paper, the analysis was based on the effect of temperature on PRR (Luz and Meiboom, 1964), and the conclusion reached was that the proton relaxation rate of the enzyme-MnADP-creatine complex was determined by the rate of exchange of water between the bulk of the solution and water in the first

hydration sphere of the Mn^{2+} ion. In contrast, this rate of exchange did not appear to be a limiting factor for the PRR of the enzyme-MnADP complex. These results could be most readily interpreted as being due to a conformational change occurring on the addition of creatine to the creatine kinase-MnADP complex. The preliminary results given here would be consistent with a similar change occurring with arginine kinase on the addition of L-arginine to the enzyme-MnADP complex. This has now been confirmed, and a more detailed analysis of the temperature effects is at present being undertaken (O'Sullivan and Marsden, personal communication).

The effect of phosphoarginine on the interaction of Mn^{2+} with arginine kinase is of interest since it indicates that an enzyme-Mn-phosphoarginine complex can be formed in the absence of nucleotides, and the formation of an enzyme-phosphoarginine complex is consistent with the enzyme having a random mechanism. Also, it is particularly noteworthy in view of the substrate inhibition by phosphoarginine discussed in Chapter IV. Since some complex of arginine kinase with Mn^{2+} and phosphoarginine is known to be formed, it is possible that a dead-end complex, enzyme-MnADP-Mn-

phosphoarginine, could also be formed. From these studies, however, it is not possible to establish the nature of the complex formed in the presence of arginine kinase, Mn^{2+} and phosphoarginine, and it would also be very difficult to determine with any certainty, from kinetic studies, that the metal complex of phosphoarginine acts as a dead-end inhibitor.

SUMMARY

1. Proton relaxation rate determinations have shown that there is very weak, and probably non-specific, binding of Mn^{2+} to arginine kinase.

2. The enhancement of PRR due to the enzyme-MnADP complex is considerably greater than that for the enzyme-MnATP complex.

3. Dissociation constants for the interaction of $MnADP^-$ and $MnATP^{2-}$ with free enzyme were determined by PRR measurements, and that for $MnADP^-$ also by kinetic methods.

4. An increase in enhancement was observed with dADP in the presence of Mn^{2+} and arginine kinase, but not with GDP.

5. A complex of enzyme-MnADP-L-arginine was demonstrated, but very little effect was observed on addition of D-arginine or creatine to the enzyme-MnADP complex.

6. The existence of a complex involving arginine kinase, Mn^{2+} and phosphoarginine was demonstrated.

CHAPTER VI

Molecular Weight, Amino Acid Analysis, Peptide Mapping and Some Preliminary Studies of the Inactivation by Iodoacetamide of the Arginine Kinase from P. longipes

INTRODUCTION

A number of studies similar to those reported in this chapter have been carried out with arginine kinases. In particular, the enzyme from the European lobster, H. vulgaris, has been investigated in some detail. The molecular weight of this enzyme was determined (Virden et al., 1966) by ultracentrifuge analysis, gel filtration and density gradient sedimentation, and the value reported was 37,000. Virden and Watts (1966a) reported the amino acid composition of this enzyme and these authors (1966b) also studied the role of thiol groups in the structure and mechanism of action of arginine kinase. Ruiz Cruz et al. (1963) had previously found that iodoacetamide inactivated the arginine kinase from H. vulgaris, and sensitivity to sulphydryl reagents had previously been reported for the arginine kinase from P. astacus (Elodi and Szorenyi, 1956) and for arginine kinase from J. verreauxi (Morrison et al., 1957). The detailed studies of Virden and Watts (1966b) indicate that there is not one single essential sulphydryl group, but that three such groups are probably essential for activity, and that the situation is complex, with one of these sulphydryl groups situated

at the active site, while two others, probably essential for activity, are involved in conformational changes in the enzyme associated with the binding of nucleotide substrates.

Pradel et al. (1965) suggest that arginine kinase from the European lobster is protected from inactivation by DTNB by the guanidino substrates, but not by the Mg-nucleotide substrates. Only three sulphydryl groups per mole reacted with DTNB. Kassab et al. (1968) have reported that this arginine kinase is inactivated by 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) and that a labelled lysine residue could be isolated after treatment of the enzyme with dansyl chloride. In addition, Pradel and Kassab (1968) have shown by reaction of the enzyme with diethyl pyrocarbonate that a histidine residue is essential for enzymic activity.

Blethen and Kaplan (1967) have studied some factors affecting the reactivation of the arginine kinase from H. americanus after its inactivation by 8 M urea, and have also determined the molecular weight by ultracentrifuge analysis, and carried out amino acid analysis on the enzyme. The molecular weight was found to be 40,000, and the reactivation of the enzyme was promoted by thiols, L-arginine and ATP.

Thoai et al. (1968) indicated that the molecular weight of the arginine kinase from the siponcle, S. nudus, is 86,500. Lacombe et al. (1969), in a study of this arginine kinase, estimated the molecular weight of the enzyme by chromatography on Sephadex G-100 in the presence and absence of 8 M urea and found the molecular weight to be 38,000 in the presence of urea, and higher in its absence.

The similarity of the amino acid compositions of the phosphagen phosphotransferases from a number of sources has been demonstrated by Thoai et al. (1968), and the amino acid compositions of a number of arginine kinases from various organisms have also been determined (Virden and Watts, 1966a; Der Terrosian et al., 1966; Blethen and Kaplan, 1967; Blethen and Kaplan, 1968). Thus, it is possible to compare the results of the amino acid analysis of the arginine kinase from P. longipes reported in this chapter with those obtained for arginine kinases from other organisms.

Although Virden and Watts (1966b) carried out a detailed study of the effects of iodoacetamide on the arginine kinase from H. vulgaris and showed that all five sulphydryl groups demonstrated by amino acid

analysis were involved in the inactivation of the enzyme by this reagent, these authors did not make use of [^{14}C]-iodoacetamide and peptide mapping to demonstrate the relative reaction of iodoacetamide with the different sulphydryl groups. It was therefore considered of interest to determine whether all the sulphydryl groups of the arginine kinase from P. longipes are reactive towards iodoacetamide, or whether the reaction of a single sulphydryl group is sufficient to cause complete inactivation. Preliminary studies of the effects of incubating arginine kinase with iodoacetamide in the absence and presence of substrates were carried out. The results of peptide mapping of tryptic digests of the protein after incubation with [^{14}C]-iodoacetamide in the absence and presence of substrates are presented in this chapter.

EXPERIMENTAL PROCEDURE

Materials

Trypsin was obtained from Worthington Biochemicals and treated with L-1-tosyl-amido-2-phenylethyl chloromethyl ketone to destroy any chymotryptic activity (Schoellman and Shaw, 1963). Iodoacetamide was obtained from Fluka, and recrystallised twice from 50% ethanol (Watts et al., 1961). Iodoacetamide solutions were prepared immediately before use. [^{14}C]-Iodoacetamide (17.2 mC/mM) was purchased from the Radiochemical Centre, Amersham, and 5,5'-dithiobis-(2-dinitrobenzoic acid), DTNB, was from Sigma and was used without further purification. All other chemicals were as described previously and arginine kinase was prepared by the method given in Chapter I.

Methods

Ultracentrifuge analysis

Ultracentrifuge analysis was carried out in a Beckman Model E analytical ultracentrifuge under the conditions given in the legend to Fig. VI.1. Prior to ultracentrifuge analysis, the enzyme was dialysed for 48 hr against several changes of 0.01 M N-ethylmorpholine-HCl buffer (pH 8.0) made to 0.1 ionic strength with KCl.

Amino acid analysis

Samples of arginine kinase (approximately 3 mg) were hydrolysed in sealed evacuated tubes in 6 M HCl at 110° for 22 hr (Crestfield et al., 1963). The HCl was then removed under reduced pressure using a rotary evaporator, and chromatographic analysis of the hydrolysate was carried out by the method of Spackman et al. (1958), using a Beckman amino acid analyser, Model 120B. The buffer flow rate was 40 ml per hr, and the height of the short column was 12 cm, and that of the long column, 50 cm. The tryptophan content of the protein was not estimated, and cysteine was determined as cysteic acid after performic oxidation of the protein. The amino acid analyses were carried out by Mr. L.B. James.

Denaturation of the enzyme for tryptic digestion

Arginine kinase was denatured by performic acid oxidation (Hirs, 1956). Performic acid was prepared by mixing 0.5 ml H_2O_2 and 9.5 ml formic acid, leaving the solution at room temperature for 2 hr, and then cooling it to 0°. The enzyme (approximately 30 mg) was dissolved in as small a volume of formic acid as possible and 5 ml of performic acid was added. After 4 hr at 0°, the protein was lyophilised, redissolved

in a small volume of formic acid, and, after addition of 10 ml of water to destroy any remaining performic acid and peroxides, the protein was again lyophilised.

Tryptic digestion

The denatured protein (approximately 25 mg) was suspended in 4 ml of a 0.5% (w/v) NH_4HCO_3 solution (pH 8.0). Trypsin was dissolved in 0.01 M HCl to inactivate any remaining chymotrypsin, and an aliquot equivalent to 1% (w/w) of the denatured protein was added. Tryptic digestion was then carried out for 4 hr at 37° and the digest lyophilised to remove the NH_4HCO_3. The tryptic peptides were redissolved in water and the pH adjusted to approximately pH 4.5 with a drop of glacial acetic acid. Very little material was insoluble at this pH and, therefore, the pH adjustment was not usually included, and samples of tryptic peptides were mapped directly, after dissolving in electrophoresis buffer.

Peptide mapping

(a) First dimension. Electrophoresis was carried out in a high voltage electrophoresis apparatus similar to that described by Ryle et al. (1955), with mineral turpentine, saturated with the appropriate electrophoresis buffer, as the inert organic coolant.

A sample of tryptic peptides (2 to 3 mg) was weighed out, dissolved in a very small volume of pyridine-acetic acid buffer (pH 4.7) (Schwartz, 1963) and applied as a 2 cm band to a strip of Whatman No. 3 MM paper. The paper was then saturated with electrophoresis buffer, excess moisture removed by blotting the strip so that it was almost dry, and electrophoresis in the pyridine-acetic acid buffer (pH 4.7) was carried out at 40 V/cm for 70 min. The paper was then allowed to dry in air.

(b) Second dimension. The strip of paper was sewn on to a fresh sheet of paper (Milstein and Sanger, 1961) and the second dimension of the peptide map involved either electrophoresis at pH 1.9 (Atfield and Morris, 1960) for 1 hr, or ascending chromatography for approximately 17 hr with (i) pyridine/isoamyl alcohol/water (35:35:30, v/v), or (ii) butanol/acetic acid/water/pyridine (30:6:24:20, v/v) as the developing solvent.

Staining for peptides

Ninhydrin. After drying the chromatograms, the peptides were detected by spraying with ninhydrin reagent, 0.1% (w/v) ninhydrin in acetone containing 0.2% (v/v) glacial acetic acid and 0.2% (v/v) pyridine.

The papers were then stored in the dark for several hours for colour to develop, and, after photography, the ninhydrin positive spots were outlined lightly in pencil before another stain was used.

Starch iodide. Larger peptides, which do not stain well with ninhydrin can be detected by chlorination followed by starch iodide treatment of the peptide map. The paper is placed in a jar with chlorine gas, produced by the reaction of $KMnO_4$ with HCl, for approximately 1 hr. The ninhydrin-positive spots disappear on such treatment. The chlorine is then removed by suspending the paper in a stream of dry air overnight, and sprayed with a 1% (w/v) solution of soluble starch containing 1% (w/v) KI (Rydon and Smith, 1952). Large peptides stain blue with this treatment.

Specific staining for histidine. Histidine-containing peptides were detected using the Pauly reagent, as modified by Milstein (personal communication to D.C. Shaw). The ninhydrin colour was removed by treatment of the chromatogram with 1% (v/v) HCl in acetone, and the following solutions were prepared and cooled on ice : A, 1% (w/v) sulphanilic acid in 0.1 M HCl; B, 5% (w/v) sodium nitrite; C, 10% (w/v) sodium

carbonate. One volume of each of A and B were chilled on ice, mixed and allowed to stand for 10 min on ice. Two volumes of C were added to the mixture and the chromatograms were immediately sprayed lightly with the solution. The histidine-containing peptides stained orange-red, while the tyrosine-containing peptides stained a purplish colour.

Inactivation of arginine kinase with iodoacetamide

The enzyme (3 mg/ml) was incubated at 30° in 0.02 M triethanolamine-HCl buffer (pH 8.0) with a 10 molar excess of iodoacetamide in the absence and presence of reactants, each at a concentration of 10 mM, and with 1 mM free Mg^{2+} present. The reaction was started by the addition of iodoacetamide and samples were taken at intervals and diluted 30-fold in 0.001 M triethanolamine-HCl buffer (pH 8.0). 5 µl samples of these diluted enzyme solutions were assayed for enzymic activity under standard conditions (Chapter I) 2 min after dilution.

In the case where [^{14}C]-iodoacetamide was used, samples were taken up to 12 min and the reaction was stopped 15 min after the addition of iodoacetamide by precipitation of the enzyme by the addition of 2 volumes of absolute ethanol, which precipitated all the

protein from solution. No control in which ethanol was added before the iodoacetamide was included. The white precipitate was centrifuged off, washed twice with 6 ml of absolute ethanol and dried overnight *in vacuo*. The protein was then performic acid oxidised, digested with trypsin and peptide maps were prepared using electrophoresis at pH 4.7 in the first dimension and ascending chromatography in pyridine/isoamyl alcohol/water (35:35:30, v/v) in the second. Radioautographs of each map were made by exposing the paper to X-ray film for 3 days. The radioactive peptides were cut out and counted as described in Chapter II.

Reaction with DTNB

Arginine kinase was treated with a 10 molar excess of DTNB at pH 8.0 in 0.1 M N-ethylmorpholine-HCl buffer, in the absence and presence of 6 M urea. Optical density readings were made at 412 mμ after 5 min and 7 min after the addition of DTNB, and the reaction was found to have gone to completion after 5 min. An optical density reading was also taken after 40 min and there was no further increase in the reading. All readings were made on a Zeiss spectrophotometer, and the molar extinction coefficient was taken to be 13,600 (Ellman, 1959).

RESULTS

Ultracentrifuge analysis

Velocity sedimentation patterns of arginine kinase, initial concentration 6 mg/ml, are shown in Fig. VI.1. The sedimentation coefficient in buffer, $S_{20,b}$, was 3.25 S. Making the usual assumptions (Fruton and Simmonds, 1958, p.41), this value suggests a molecular weight of approximately 40,000. Some material accumulated at the bottom of the cell during sedimentation (see Fig. VI.1), indicating the presence of aggregated material in the preparation. The clear arginine kinase solution from which the sample for ultracentrifuge analysis was taken became opalescent on standing at room temperature for the duration of the ultracentrifuge run. This is also consistent with the aggregation of the protein under the prevailing conditions.

Amino acid analysis

The results of amino acid analysis of the arginine kinase from *P. longipes* are given in Table VI.1 and are the mean values from four separate amino acid analyses. The values and standard errors are given and the number of residues was rounded off to the nearest whole number for comparison with the amino

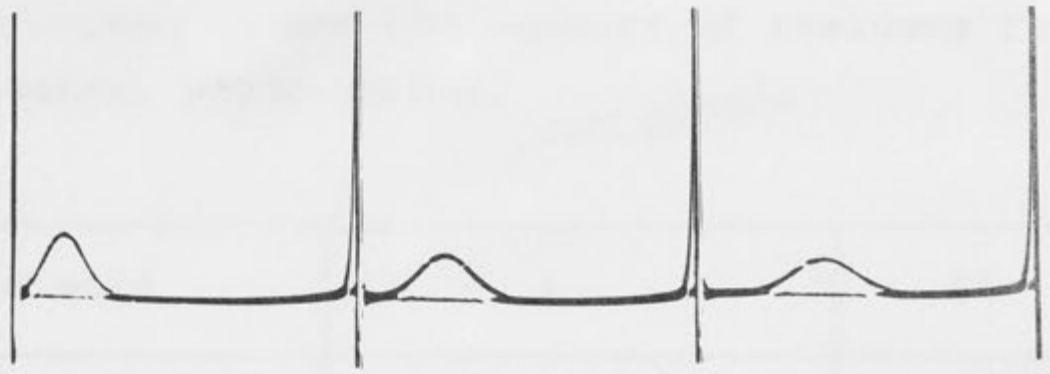


Fig. VI.1. Ultracentrifuge patterns obtained with a sample of purified arginine kinase. The analysis was carried out in 0.01 M N-ethylmorpholine-HCl buffer (pH 8.0) made up to an ionic strength of 0.1 with KCl, at a temperature of 21.9°, using a Beckman Model E Analytical ultracentrifuge. The initial protein concentration was 6 mg/ml. The rotor speed was 50,740 rev/min, and the rotor type was AnH. A 12 mm double sector cell with filled Epon centrepiece was used. Schlieren optics were used with a 70° phase plate angle and the pictures were taken at 35 min, 67 min and 99 min after reaching full speed.

The analysis was carried out by Dr. J.R. Dunstone.

Table VI.1.

Amino acid composition of arginine kinase purified from P. longipes. The values given in column I and the weighted means of the results of four analysis, with the standard errors. Cysteine was determined as cysteic acid after performic acid oxidation of the enzyme. The values given in column II are the numbers of residues rounded off to the nearest whole number.

Amino acid	I	II
Lysine	29.35 ± 1.12	29
Histidine	8	8
Arginine	17.31 ± 0.52	17
Aspartate	33.18 ± 1.10	33
Threonine	16.90 ± 0.32	17
Serine	17.11 ± 0.62	17
Glutamate	44.08 ± 0.82	44
Proline	12.34 ± 0.79	12
Glycine	25.89 ± 1.56	26
Alanine	23.63 ± 0.77	24
Cysteine	4.94 ± 0.11	5
Valine	20.16 ± 1.08	20
Methionine	8.29 ± 0.26	8
Isoleucine	16.94 ± 0.57	17
Leucine	28.3 ± 1.49	28
Tyrosine	9.80 ± 0.06	9
Phenylalanine	17.83 ± 0.40	18

acid compositions of other arginine kinases. Cysteine was determined as cysteic acid after performic acid oxidation of the protein. The values presented were calculated on the basis of 8 histidine residues per mole. The minimum chemical molecular weight calculated from the number of amino acids and their respective residue weights (molecular weight minus water) is approximately 38,000, but cannot be determined exactly since the tryptophan content was not determined.

Peptide mapping

A peptide map obtained after electrophoresis at pH 4.7, followed by ascending chromatography in pyridine/isoamyl alcohol/water (35:35:30, v/v) is shown in Fig. VI.2. The very small quantity of material which was insoluble at pH 4.5 was not removed before electrophoresis, since there was no detectable difference between peptide maps obtained in the presence and absence of this "core" material. No peptides which were not apparent after ninhydrin treatment could be detected on spraying the chromatogram with the starch-iodide reagent, indicating that there are no ninhydrin-negative or very large peptides. Starch iodide treatment of a peptide map of the pH 4.5 insoluble material, prepared by electrophoresis at pH 8.9, followed by ascending chromatography

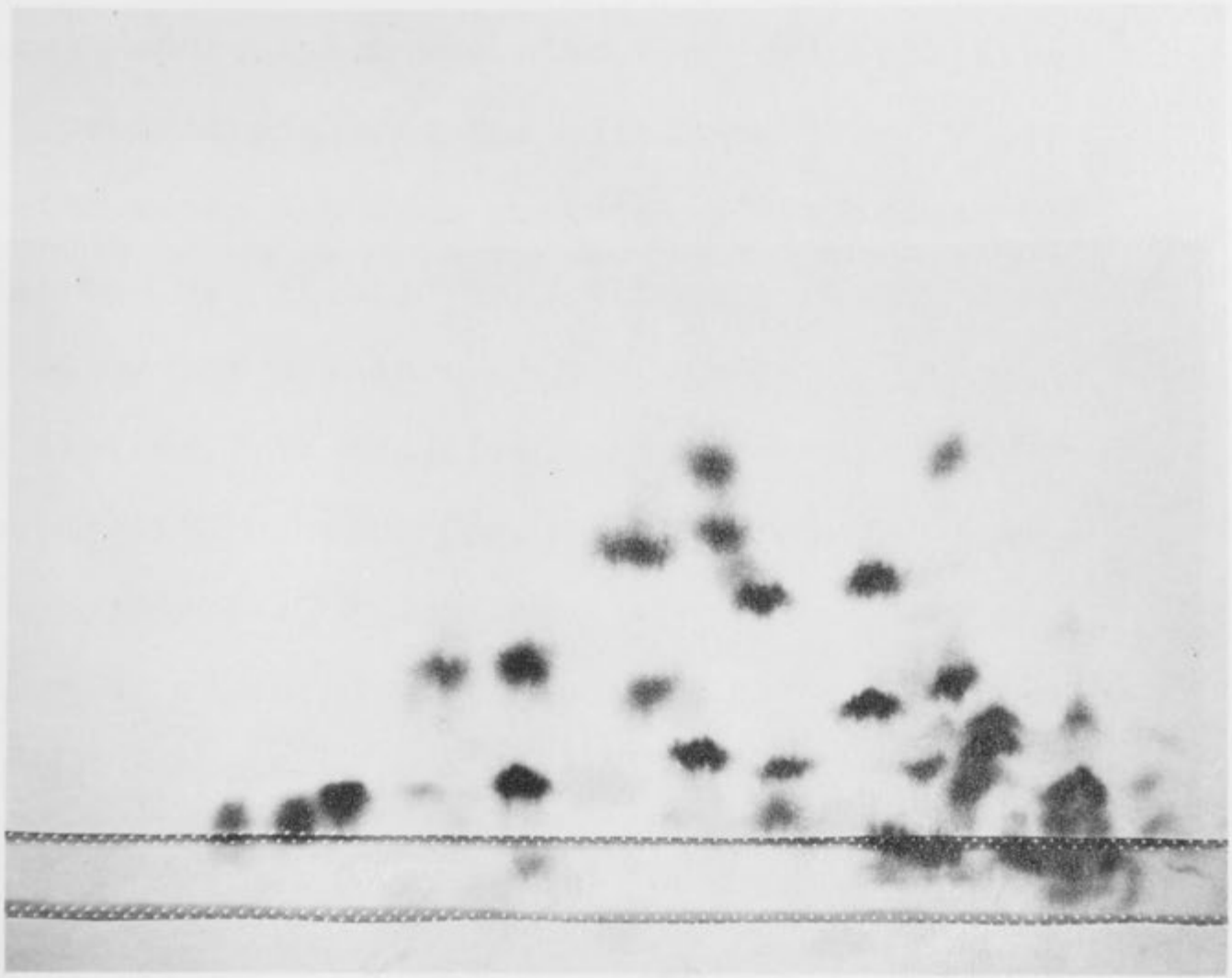


Fig. VI.2. Peptide map of arginine kinase.

1st dimension : electrophoresis at pH 4.7, 40 V/cm for 70 min.

2nd dimension : ascending chromatography with pyridine/isoamyl alcohol/water (35:35:30, v/v) for 17 hr.

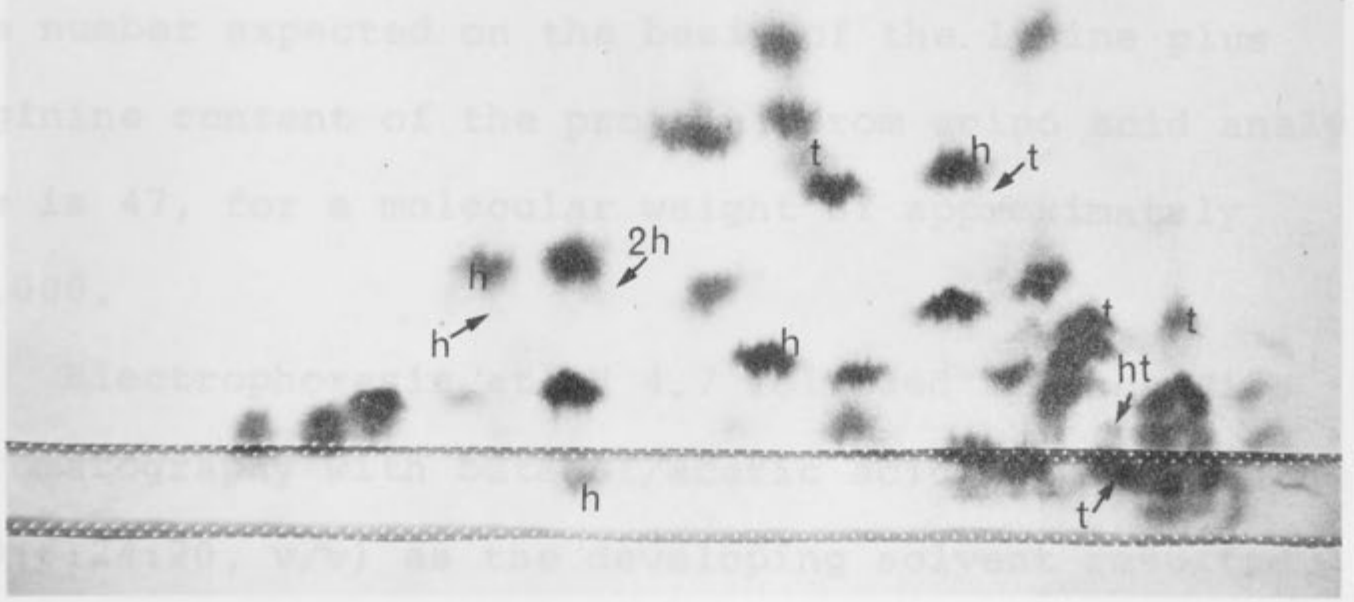


Fig. VI.3. Peptide map of arginine kinase as shown in Fig. VI.2, with histidine-containing peptides marked h and tyrosine-containing peptides marked t.

in the pyridine/isoamyl alcohol/water solvent, revealed a single spot, which was also ninhydrin-positive.

Specific staining for histidine (Fig. VI.3) revealed seven peptides containing histidine, and, of these, one was stained more strongly than the others, indicating the presence of two histidine residues in that peptide. In addition, six spots staining for tyrosine with varying degrees of colour were detected. The total number of peptides detected was 44 to 51, and the number expected on the basis of the lysine plus arginine content of the protein, from amino acid analysis is 47, for a molecular weight of approximately 40,000.

Electrophoresis at pH 4.7 followed by ascending chromatography with butanol/acetic acid/water/pyridine (30:6:24:20, v/v) as the developing solvent resulted in the peptide map shown in Fig. VI.4. Again there is good separation of the peptides and approximately 40 peptides can be detected, but it is more difficult to distinguish individual peptides near the origin than in Fig. VI.3, so that the pyridine/isoamyl alcohol/water chromatography solvent was generally used.

Fig. VI.4. Peptide map of arginine kinase.

1st dimension : electrophoresis at pH 4.7, 40 V/cm for 70 min.

2nd dimension : ascending chromatography with butanol/acetic acid/water/pyridine (30:6:24:20, v/v) for 17 hr.

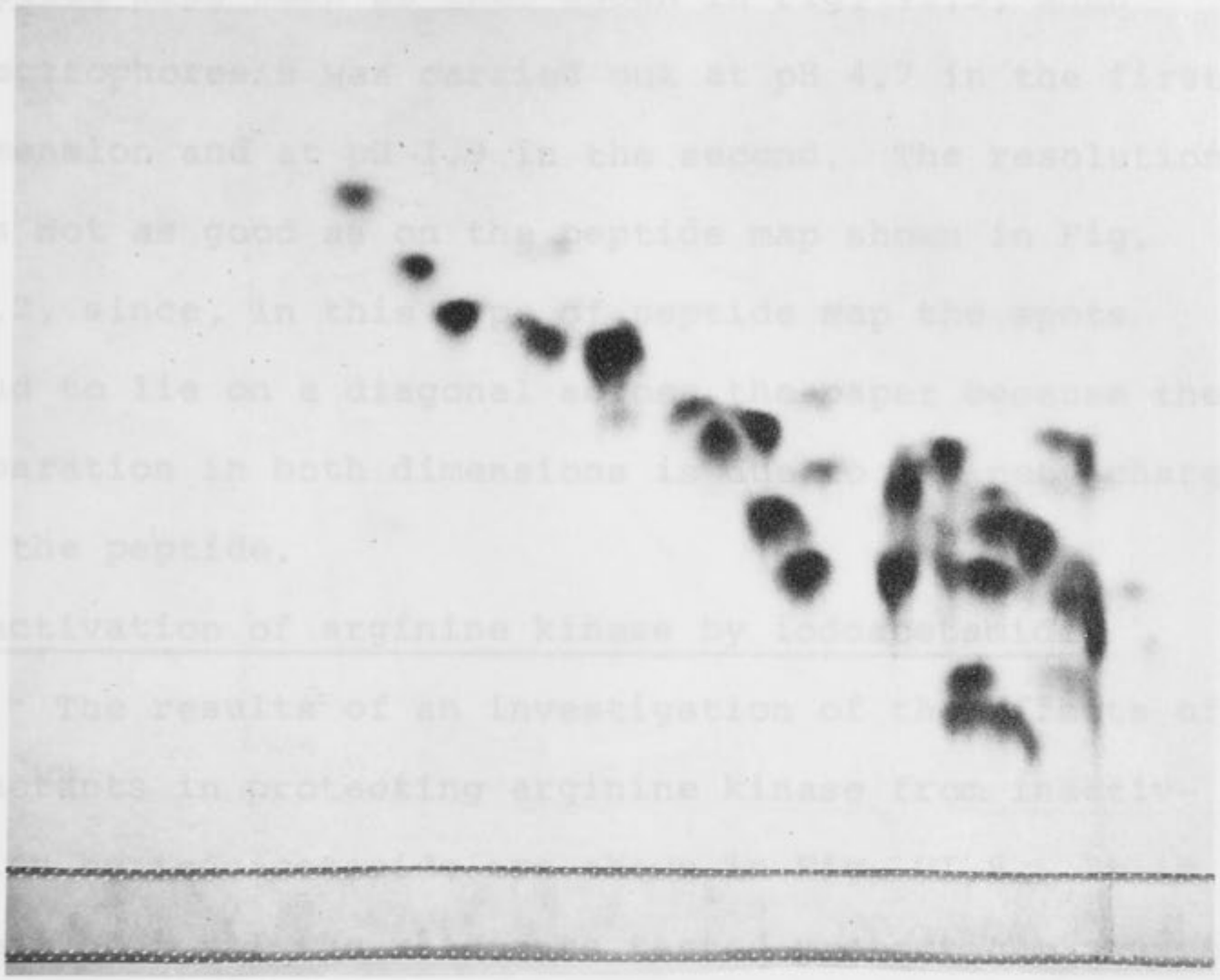


Fig. VI.5. Peptide map of arginine kinase.

1st dimension : electrophoresis at pH 4.7, 40 V/cm for 70 min.

2nd dimension : electrophoresis at pH 1.9, 40 V/cm for 60 min.

Two-dimensional electrophoresis resulted in peptide maps such as that shown in Fig. VI.5, when electrophoresis was carried out at pH 4.7 in the first dimension and at pH 1.9 in the second. The resolution was not as good as on the peptide map shown in Fig. VI.2, since, in this type of peptide map the spots tend to lie on a diagonal across the paper because the separation in both dimensions is due to the nett charge on the peptide.

Inactivation of arginine kinase by iodoacetamide

The results of an investigation of the effects of reactants in protecting arginine kinase from inactivation by iodoacetamide are shown in Fig. VI.6. It is clear that all the reactants tested protect the enzyme from inactivation by iodoacetamide to some extent, but that arginine is much more effective in this than the nucleotide substrates. The presence of $MgADP^-$ as well as arginine, allowing the formation of a dead-end complex enzyme-MgADP-arginine, further increases the protection, and the presence of both $MgATP^{2-}$ and arginine, which give rise to all reactants in equilibrium concentrations, gives slightly greater protection from inactivation by iodoacetamide.

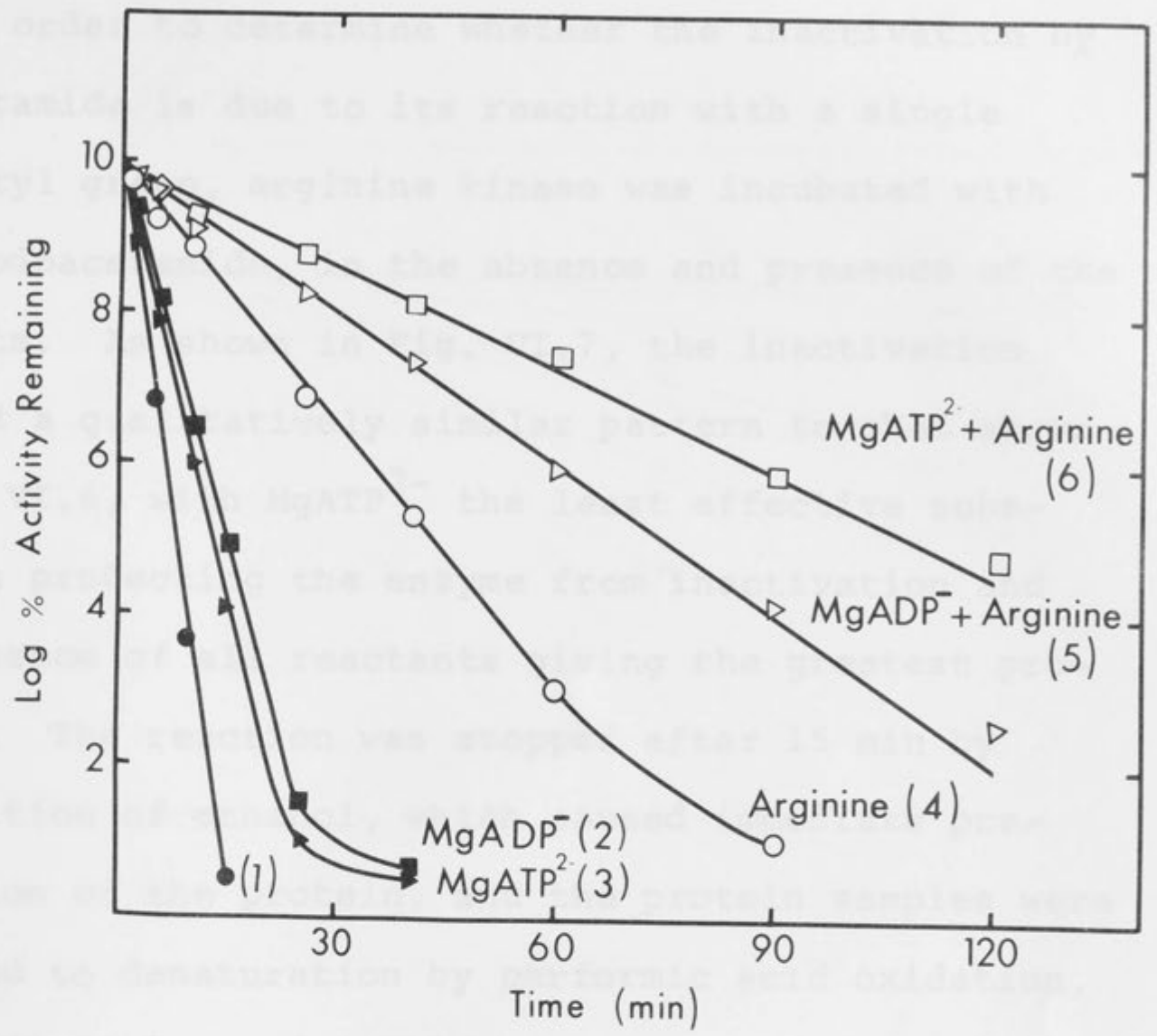


Fig. VI.6. First-order log plots of the effects of reactants on the inactivation of arginine kinase by iodoacetamide.

Arginine kinase was incubated at 30° with a 10 molar excess of iodoacetamide in the absence (1) and in the presence of reactants. Samples (2), (3) and (4) contained 10 mM $MgADP^-$, $MgATP^{2-}$ and arginine, respectively, and samples (5) and (6) contained 5 mM $MgADP^-$ + 5 mM arginine, and 5 mM $MgATP^{2-}$ + 5 mM arginine, respectively.

In order to determine whether the inactivation by iodoacetamide is due to its reaction with a single sulphydryl group, arginine kinase was incubated with [^{14}C]-iodoacetamide, in the absence and presence of the reactants. As shown in Fig. VI.7, the inactivation followed a qualitatively similar pattern to that shown in Fig. VI.6, with $MgATP^{2-}$ the least effective substrate in protecting the enzyme from inactivation and the presence of all reactants giving the greatest protection. The reaction was stopped after 15 min by the addition of ethanol, which caused immediate precipitation of the protein, and the protein samples were subjected to denaturation by performic acid oxidation, tryptic digestion and peptide mapping. After staining the peptide maps with ninhydrin and photographing them, a radioautograph of each map was made. Each of these showed five strongly radioactive spots and the corresponding areas of the peptide maps were cut out and the radioactivity of each determined using the scintillation counter. The peptide map obtained after iodoacetamide inactivation of arginine kinase in the absence of reactants is shown in Fig. VI.8, and the positions of the radioactive spots on this peptide map in Fig. VI.9. The positions of the radioactive

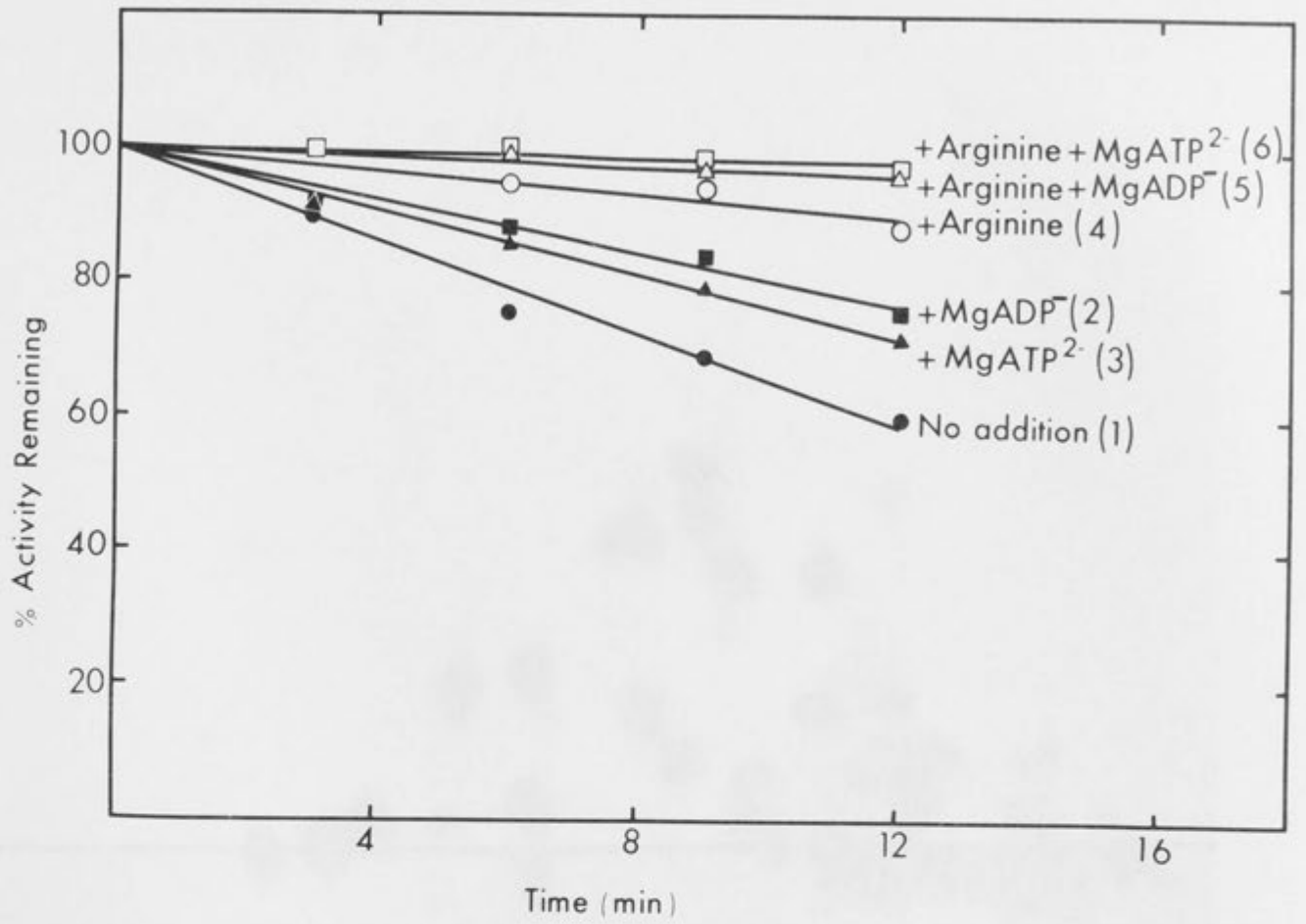


Fig. VI.7. Inactivation of arginine kinase by [^{14}C]-iodoacetamide in the absence and presence of reactants.

Arginine kinase was incubated at 30° with a 10 molar excess of [^{14}C]-iodoacetamide in the absence and presence of iodoacetamide. The concentrations of reactants in the samples were as described in the legend to Fig. VI.6.

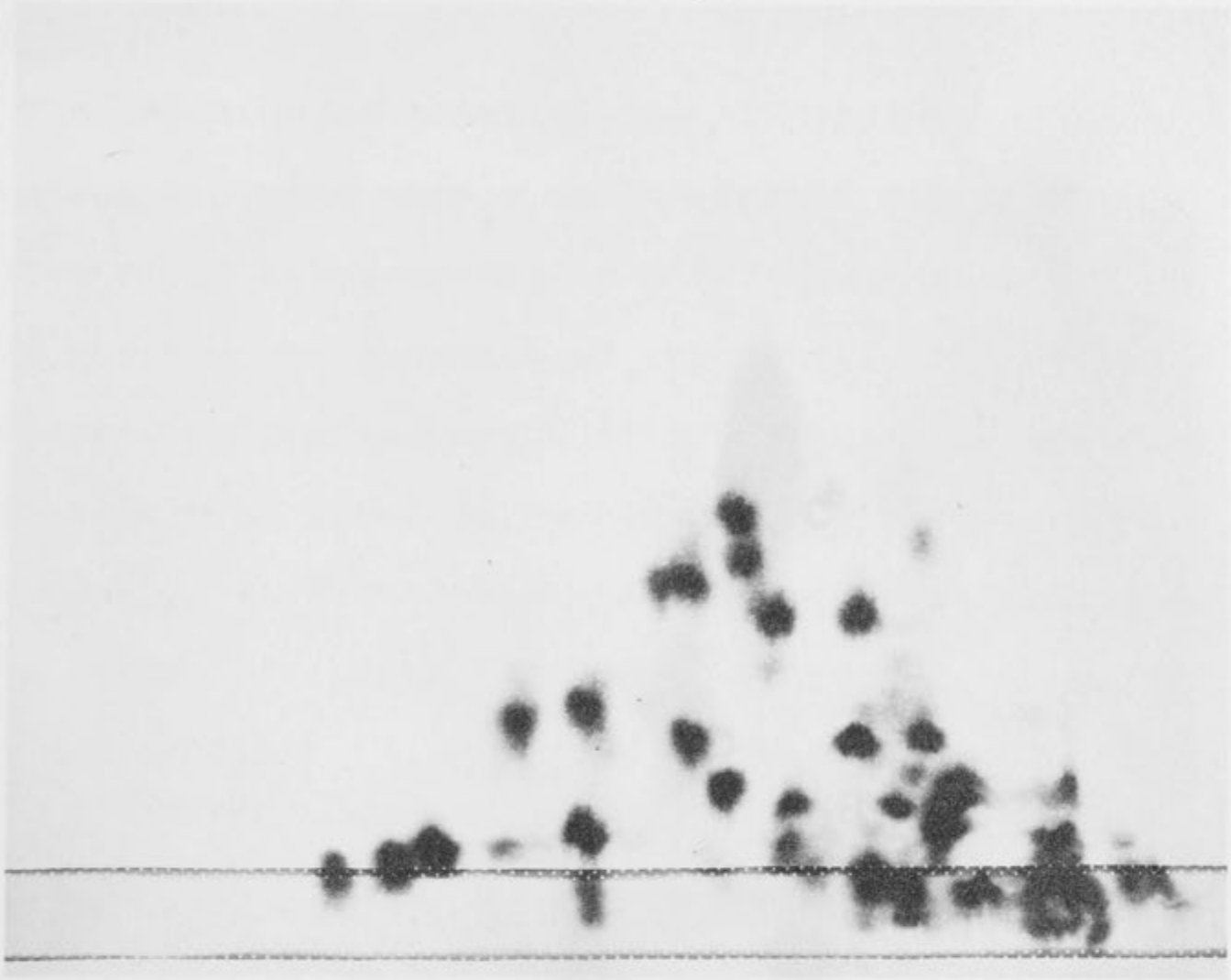


Fig. VI.8. Peptide map of arginine kinase after incubation with [^{14}C]-iodoacetamide for 15 min in the absence of reactants.

1st dimension : electrophoresis at pH 4.7, 40 V/cm for 70 min.

2nd dimension : ascending chromatography with pyridine/isoamyl alcohol/water (35:35:30, v/v) for 17 hr.

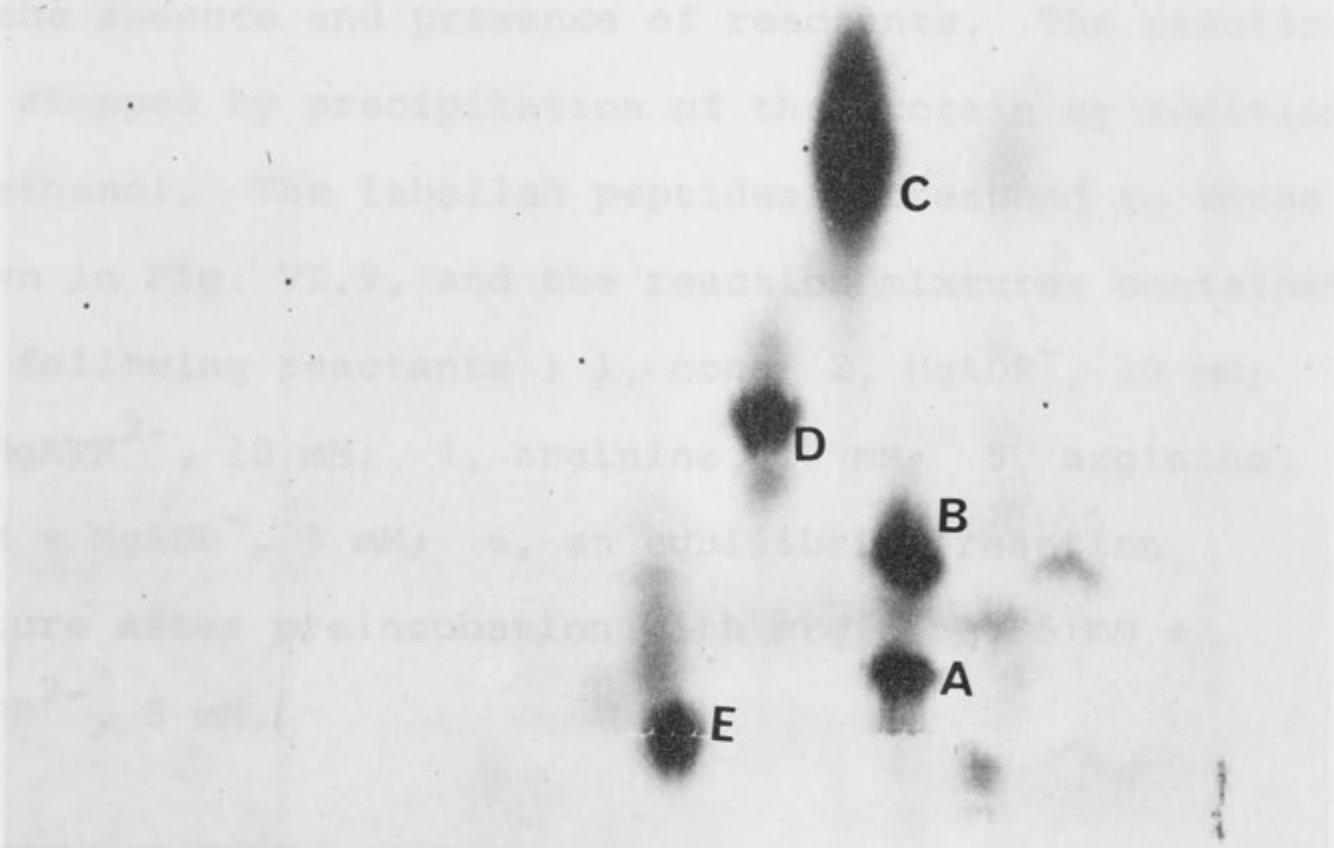


Fig. VI.9. Radioautograph of the peptide map shown in Fig. VI.8 with the radioactive peptides marked A, B, C, D and E for reference with Table VI.2.

Table VI.2.

The relative radioactivities of peptides after incubating arginine kinase at 30° for 15 min in the presence of 10 molar excess of [^{14}C]-iodoacetamide in the absence and presence of reactants. The reaction was stopped by precipitation of the protein by addition of ethanol. The labelled peptides correspond to those shown in Fig. VI.9, and the reaction mixtures contained the following reactants : 1, none; 2, $MgADP^-$, 10 mM; 3, $MgATP^{2-}$, 10 mM; 4, arginine, 10 mM; 5, arginine, 5 mM + $MgADP^-$, 5 mM; 6, an equilibrium reaction mixture after preincubation with arginine, 5 mM + $MgATP^{2-}$, 5 mM.

Peptide map	Radioactivity (cpm)				
	Peptide A	Peptide B	Peptide C	Peptide D	Peptide E
1	3,692	4,060	28,598	5,429	4,133
2	2,463	1,913	13,910	1,817	1,954
3	1,338	1,333	11,326	1,094	1,326
4	2,800	3,773	11,471	2,765	2,309
5	1,928	1,495	4,049	1,470	1,755
6	760	890	2,825	558	690

spots were the same on all the peptide maps, but did not correspond well to particular ninhydrin-positive spots. This may be because, after carboxymethylation of a residue in a peptide, the peptide would move to a position different from that of the same peptide containing cysteic acid.

The relative radioactivities of the labelled spots from the peptide maps are given in Table VI.2, and indicate that spot C (Fig. VI.9) is more heavily labelled than any other, even when there is a high degree of protection by reactants.

Reaction of arginine kinase with DTNB

The reaction of arginine kinase with DTNB indicated that, assuming a molecular weight of 38,000 for the enzyme, 3.06 sulphydryl groups are present per mole of enzyme. However, after treatment with 6 M urea, reaction with DTNB indicated the presence of 4.26 sulphydryl groups per mole of arginine kinase. This suggests that treatment of the enzyme with urea has caused denaturation of the enzyme such that one more sulphydryl group is available to react with DTNB. However, amino acid analysis and inactivation studies with iodoacetamide show the existence of 5 sulphydryl groups per mole of enzyme, and it is possible that one of these is inaccessible for reaction with DTNB even after urea treatment.

DISCUSSION

The molecular weight of approximately 40,000, as determined for the arginine kinase from P. longipes by sedimentation velocity ultracentrifuge analysis, is in the range observed for the arginine kinases from a number of crustacean sources. Some of these are given in Table VI.3. On the basis of a molecular weight of approximately 40,000, the amino acid analysis shown in Table VI.1 is obtained, and, again, this is very similar to the amino acid compositions of arginine kinases from other Crustacea (Table VI.4). Thus, it appears that there is a fairly high degree of homology between the arginine kinases of Crustacea.

The peptide maps obtained in this study are consistent with the enzyme having a minimum chemical molecular weight of approximately 38,000. The amino acid composition determined assuming this molecular weight gives a total of 46 arginine and lysine residues and this would yield 47 peptides on tryptic digestion. The number of tryptic peptides detected is between 44 and 51. Although the peptide maps cannot be compared directly with those obtained by other groups of workers, it is of interest to note that Kassab et al. (1967) obtained 45 to 48 peptides after tryptic digestion of

Table VI.3.

Molecular weights of arginine kinases from a number of crustaceans.

Crustacean	Molecular weight of arginine kinase	References
Homarus vulgaris (European lobster)	37,000 43,000	Virden et al. (1966) Der Terrossian et al. (1966)
Palinurus vulgaris (European sea-water crayfish)	39,000	Moreland et al. (1967)
Carcinus maenas (Shore crab)	38,000	Moreland et al. (1967)
Potamobius astacus (Fresh-water crayfish)	43,000	Elodi and Szorenyi (1956)
Homarus americanus (American lobster)	40,000	Blethen and Kaplan (1967)
Callinectus sapidus (Blue crab)	37,000	Blethen and Kaplan (1968)
Pagurus bernhardus (Hermit crab)	38,000	Blethen and Kaplan (1968)
Limulus polyphemus (Horseshoe crab)	38,000	Blethen and Kaplan (1968)

Table VI.4.

The amino acid compositions of arginine kinases from a number of crustaceans.

Amino acid	*Panulirus longipes* (W.Australian crayfish)	*Homarus vulgaris* (European lobster) (a)	(b)	*Homarus americanus* (American lobster) (c)	*Callinectus sapidus* (Blue crab) (d)	*Pagurus bernhardus* (Hermit crab) (d)
Lysine	29	28	31	27	20	32
Histidine	8	8	8	8	8	6
Arginine	17	16	19	16	15	15
Aspartate	33	31	38	38	37	36
Threonine	17	18	21	17	20	21
Serine	17	18	20	16	16	20
Glutamate	44	39	46	39	41	33
Proline	12	13	11	12	11	13
Glycine	26	27	28	28	28	26
Alanine	24	24	28	25	21	23
Cysteine	5	5	6	5	5	5
Valine	20	18	22	22	23	19
Methionine	8	8	8	8	7	7
Isoleucine	17	17	20	17	13	13
Leucine	28	31	33	28	32	29
Tyrosine	10	10	11	9	11	9
Phenylalanine	18	18	20	19	15	18
Tryptophan	-	2	2	3	2	2

(a) Virden and Watts (1966a)
(b) Der Terrossian et al. (1966)
(c) Blethen and Kaplan (1967)
(d) Blethen and Kaplan (1968)

arginine kinase from the European lobster and Lacombe et al. (1969) detected 49 tryptic peptides on peptide maps of the arginine kinase from the siponcle, S. nudus. This enzyme has a molecular weight of 86,500 (Thoai et al., 1968) and, from the amino acid composition of the enzyme, the number of tryptic peptides expected is approximately 100. Thus, it seems probable that this arginine kinase is composed of two identical subunits, each of molecular weight approximately 40,000. Forty six tryptic peptides were observed after tryptic digestion of creatine, taurocyamine and lombricine kinases (Kassab et al., 1967), although these enzymes also have molecular weights in the region of 80,000 (Thoai et al., 1965).

The presence of seven histidine-containing peptides, shown in Fig. VI.3, is in good agreement with the eight histidines determined on amino acid analysis of the arginine kinase from P. longipes, assuming a molecular weight of approximately 40,000, especially since it is likely that one of these peptides contains two histidine residues. Pradel and Kassab (1968) have reported the existence of a histidine residue essential for activity in the arginine kinase of the European lobster.

Blethen and Kaplan (1968), in their comparative study of arthropod arginine kinases, found by immuno-diffusion techniques using rabbit antiserum to the arginine kinases from the blue crab and the American lobster that there was considerable cross-reaction with arginine kinases from other Crustacea. This is another indication of the high level of similarity between these enzymes.

Pradel et al. (1965), in a study of the arginine kinase from the European lobster, observed the reaction of three sulphydryl groups with DTNB, but subsequent studies by Kassab et al. (1967) showed the reaction of six sulphydryl groups of arginine kinase with DTNB, assuming the molecular weight to be 43,000, both in the presence and absence of urea. The reaction of the arginine kinase from P. longipes with DTNB indicated the presence of three free sulphydryl groups in the absence of a denaturing agent and four in the presence of 6 M urea. Thus, denaturation of the protein appears to make available for reaction with DTNB one sulphydryl group which was inaccessible in the native enzyme. Five strongly radioactive peptides were observed on peptide maps after reaction of the enzyme with [^{14}C]-iodoacetamide, and amino acid analysis also suggests the existence of five half-cystine residues per mole

of enzyme. It is possible, if two sulphydryl groups are close together on the polypeptide chain, that the reaction of one of these with DTNB could, by steric effects, protect the other from reacting with DTNB, because of the 5-thio-2-nitrobenzoic acid attached to the first.

The preliminary results obtained with iodoacetamide are similar to those obtained by Virden and Watts (1966b). These authors carried out a detailed study of the inactivation of the arginine kinase from H. vulgaris by iodoacetamide and concluded that the molecule contains five reactive thiol groups per 37,000 molecular weight. The situation revealed by these investigations appears to be complex, and there does not seem to be one single sulphydryl group essential for enzymic activity. However, although these authors indicated that all five sulphydryl groups reacted with iodoacetamide to inactivate the enzyme, they did not show by peptide mapping of the iodoacetamide-inactivated enzyme that five peptides were labelled. The results of inactivation of the arginine kinase from P. longipes by iodoacetamide in the absence and presence of substrates (Fig. VI.6) show marked similarities to those presented by Virden and Watts (1966b). The Mg-nucleotide complexes give slight protection from

inactivation by iodoacetamide, and arginine much greater protection. In the presence of arginine and $MgADP^-$, when the dead-end complex, enzyme-MgADP-arginine, is probably formed there is greater protection than with arginine alone. Both the results presented here, and those given by Virden and Watts (1966b), show that the presence of all reactants in equilibrium concentrations affords the greatest protection against iodoacetamide inactivation.

A preliminary study of the inactivation of arginine kinase by [^{14}C]-iodoacetamide in the absence and presence of reactants was carried out to determine whether the protection observed with the nucleotide and guanidino substrates could be attributed to their interaction with particular sulphydrvl groups. The reactions were stopped when approximately 50% of the original activity remained in the arginine kinase preparation in the absence of reactants, and virtually no inactivation had occurred in the samples containing arginine and $MgADP^-$ or an equilibrium mixture of reactants with the enzyme. In all cases, however, peptide maps showed five heavily labelled radioactive peptides, indicating that there is not one single sulphydryl group essential for activity. The results are consistent with those reported by Virden and Watts

(1966b), suggesting that the inactivation of the arginine kinases from H. vulgaris and P. longipes occurs by a similar mechanism. The relative radioactivities of the labelled peptides (Fig. VI.9) are given in Table VI.2, but no detailed quantitative conclusions can be drawn from these results. It does appear, however, that the interaction of substrates with the enzyme may cause some conformational changes in the protein structure, so that all the sulphydryl groups are less readily available for reaction with iodoacetamide.

The linearity of the log plot of inhibition against time (Fig. VI.6) indicates at first sight that all five sulphydryl groups are equivalent, or that only one reacts with iodoacetamide. However, from the results of peptide mapping of [^{14}C]-iodoacetamide-inactivated arginine kinase (Figs. VI.8 and VI.9), it is apparent that neither of these alternatives holds. From the unequal labelling of the peptides (Table VI.2) it seems probable that reaction of iodoacetamide with the first sulphydryl group, that in peptide C of Table VI.2, is rate-limiting. This could indicate that the sulphydryl group of peptide C is the only oneessential for activity. When iodoacetamide reacts with this group, some conformational

change may take place, allowing the other sulphydryl groups to react with iodoacetamide at a greater rate than does the first. The effects of substrates in protecting the enzyme against inactivation by iodoacetamide indicate that the guanidino substrate combines with arginine kinase in such a way as to hinder the reaction of the first molecule of iodoacetamide, but, when this has taken place, the other sulphydryl groups become available for reaction, so that first order log plots are obtained. The nucleotide substrates appear to function in a similar way, but are not nearly as effective as arginine in protecting the enzyme against inactivation by iodoacetamide.

Thus it appears that, although no detailed conclusions can be drawn from these preliminary investigations of the inactivation of arginine kinase from P. longipes by iodoacetamide, the process probably occurs by a mechanism similar to that for the enzyme from H. vulgaris. Also, it is interesting to note the marked similarities in the molecular weights, amino acid compositions and the inactivation by iodoacetamide observed with the arginine kinases from the European lobster, Homarus vulgaris, and the West Australian sea-water crayfish, Panulirus longipes.

SUMMARY

1. The molecular weight of the arginine kinase from P. longipes was estimated by sedimentation velocity ultracentrifugation and found to be approximately 40,000.

2. Amino acid analysis of this arginine kinase was carried out and the amino acid composition was found to be very similar to those of arginine kinases from other Crustacea. The minimum chemical molecular weight was 38,000.

3. Peptide mapping of tryptic digests of the protein gave 44 to 51 peptides, indicating that the enzyme is not composed of identical subunits.

4. Inactivation of the enzyme by iodoacetamide was consistent with the existence of five sulphydryl groups per mole reactive with iodoacetamide, since five radioactive tryptic peptides could be detected on peptide maps prepared after reaction of the enzyme with [^{14}C]-iodoacetamide. Arginine gave more effective protection against inactivation than did the Mg-complexes of nucleotides.

5. DTNB reacted with the enzyme and the results indicated the presence of three reactive sulphydryl groups per mole in the native enzyme, and four in the presence of 6 M urea.

BIBLIOGRAPHY

Alberty, R.A. (1953). J. Am. Chem. Soc., 75, 1928.

Alberty, R.A. (1958). J. Am. Chem. Soc., 80, 1777.

Atfield, G.N. and Morris, C.J.O.R. (1960). Biochem. J., 74, 37P.

Baldwin, E. and Yudkin, W.H. (1950). Proc. Roy. Soc. (London), B, 136, 614.

Berger, L. (1956). Biochim. Biophys. Acta, 20, 23.

Bessman, S.P. and Fonyo, A. (1966). Biochem. Biophys. Res. Commun., 22, 597.

Blethen, S.L. and Kaplan, N.O. (1967). Biochemistry, 6, 1413.

Blethen, S.L. and Kaplan, N.O. (1968). Biochemistry, 7, 2123.

Bloembergen, N. and Morgan, L.O. (1961). J. Chem. Phys., 34, 842.

Bock, R.M. (1960). In "The Enzymes", Vol. II, p.3. Ed. by Boyer, P.D., Lardy, H. and Myrbäck, K. Academic Press : New York and London.

Bock, R.M., Ling, N.-S., Morell, S.A. and Lipton, S.H. (1956). Arch. Biochem. Biophys., 62, 253.

Bodenstein, M. (1913). Z. Physik. Chem., 85, 329, see Segal (1959).

Boyer, P.D. (1959). Arch. Biochem. Biophys., 82, 387.

Boyer, P.D. (1962). In "The Enzymes", Vol. VI, p.95. Ed. by Boyer, P.D., Lardy, H. and Myrbäck, K. Academic Press : New York and London.

Boyer, P.D. and Bieber, L.L. (1967). In "Methods in Enzymology", Vol. X, p.768. Ed. by Estabrook, R.W. and Pullman, M.E. Academic Press, N.Y.

Bradbury, E.M. and Crane-Robinson, C. (1968). Nature, 220, 1079.

Briggs, G.E. and Haldane, J.B.S. (1925). Biochem. J., 19, 338.

Brown, A.J. (1902). J. Chem. Soc., 81, 373.

Bücher, T. and Pfleiderer, G. (1955). In "Methods in Enzymology", Vol. I, p.435. Ed. by Colowick, S.P. and Kaplan, N.O. Academic Press, N.Y.

Carlson, F.D. and Siger, A. (1960). J. Gen. Physiol., 44, 33.

Carr, H.Y. and Purcell, E.M. (1954). Phys. Rev., 73, 679.

Cleland, W.W. (1963a). Biochim. Biophys. Acta, 67, 104.

Cleland, W.W. (1963b). Biochim. Biophys. Acta, 67, 173.

Cleland, W.W. (1963c). Biochim. Biophys. Acta, 67, 188.

Cleland, W.W. (1963d). Nature, 198, 463.

Cleland, W.W. (1967). Ann. Rev. Biochem., 36, 77.

Cohn, M. (1959). J. Cell. Comp. Physiol., 54, Suppl. 1, 17.

Cohn, M. (1963). Biochemistry, 2, 623.

Cohn, M. (1967). In "Magnetic Resonance in Biological Systems", p.101. Ed. by Ehrenberg, E., Malmström, B.G. and Vänngård, T. Pergamon Press, Oxford.

Cohn, M. and Leigh, J.S. (1962). Nature, 193, 1037.

Colomb, M.G., La Turaze, J.G. and Vignais, P.V. (1966). Biochem. Biophys. Res. Commun., 24, 909.

Crestfield, A.M., Moore, S. and Stein, W.H. (1963). J. Biol. Chem., 238, 622.

Dakin, W.J. (1952). In "Australian Seashores", p.183. Angus and Robertson, Sydney.

Davis, B.J. (1964). Ann. N.Y. Acad. Sci., 121, 404.

Der Terrossian, E., Kassab, R., Pradel, L.A. and Thoai, N.v. (1966). Biochim. Biophys. Acta, 122, 462.

Doudoroff, M., Barker, H.A. and Hassid, W.Z. (1947). J. Biol. Chem., 168, 725.

Dowd, J.E. and Riggs, D.S. (1965). J. Biol. Chem., 240, 863.

Dowler, M.J. and Nakada, H.I. (1968). J. Biol. Chem., 243, 1434.

Eisinger, J., Shulman, R.G. and Blumberg, W.E. (1961). Nature, 192, 963.

Eisinger, J., Shulman, R.G. and Szymanski, B.M. (1962). J. Chem. Phys., 36, 1721.

Ellman, G.L. (1959). Arch. Biochem. Biophys., 82, 70.

Elodi, P. and Szorenyi, E.T. (1956). Acta Physiol. Hung., 9, 367.

Ennor, A.H. and Morrison, J.F. (1958). Physiol. Rev., 38, 631.

Ennor, A.H. and Stocken, L.A. (1948). Biochem. J., 43, 190.

Florini, J.R. and Vestling, C.S. (1957). Biochim. Biophys. Acta, 25, 575.

Fromm, H.J. (1969). Europ. J. of Biochem., 7, 385.

Fromm, H.J. and Nelson, D.R. (1962). J. Biol. Chem., 237, 215.

Fromm, H.J., Silverstein, E. and Boyer, P.D. (1964). J. Biol. Chem., 239, 3645.

Fromm, H.J. and Zewe, V. (1962). J. Biol. Chem., 237, 3027.

Fruton, J.S. and Simmonds, S. (1958). In "General Biochemistry". John Wiley and Sons, New York.

Goffeau, A., Pedersen, P.L. and Lehninger, A.L. (1967). J. Biol. Chem., 242, 1845.

Gornall, A.G., Bardawill, C.J. and Davidson, M.M. (1949). J. Biol. Chem., 177, 751.

Griffiths, D.E., Morrison, J.F. and Ennor, A.H. (1957). Biochem. J., 65, 153.

Grinnell, F.L. and Nishimura, J.S. (1969). Biochemistry, 8, 562.

Grunnet, N. and Lundquist, F. (1967). Europ. J. of Biochem., 3, 78.

Gulbinsky, J.S. and Cleland, W.W. (1968). Biochemistry, 7, 566.

Hammes, G.G. and Kochavi, D. (1962a). J. Am. Chem. Soc., 84, 2069.

Hammes, G.G. and Kochavi, D. (1962b). J. Am. Chem. Soc., 84, 2073.

Hanson, J. and Lowy, J. (1960). In "Structure and Function of Muscle", Vol. I, p.265. Ed. by Bourne, G.H. Academic Press, N.Y.

Hanson, T.L. and Fromm, H.J. (1965). J. Biol. Chem., 240, 4133.

Hass, L.F., Boyer, P.D. and Reynard, A.M. (1961). J. Biol. Chem., 236, 2284.

Henderson, J.F., Brox, L.W., Kelley, W.N., Rosenbloom, F.M. and Seegmiller, J.E. (1968). J. Biol. Chem., 243, 2514.

Henri, V. (1903). In "Lois generales de l'action des diastases", Hermann, Paris. See Segal (1959).

Henson, C.P. and Cleland, W.W. (1964). Biochemistry, 3, 338.

Hirs, C.H.W. (1956). J. Biol. Chem., 219, 611.

Hori, M. and Henderson, J.F. (1966). J. Biol. Chem., 241, 3404.

Hunter, A. (1928). In "Creatine and Creatinine", Longmans, London.

James, E. and Morrison, J.F. (1966a). J. Biol. Chem., 241, 4758.

James, E. and Morrison, J.F. (1966b). Biochim. Biophys. Acta, 128, 327.

Kaji, A. and Colowick, S.P. (1965). J. Biol. Chem., 240, 4454.

Kassab, R., Pradel, L.A., Der Terrossian, E. and Thoai, N.v. (1967). Biochim. Biophys. Acta, 132, 347.

Kassab, R., Roustan, C. and Pradel, L.A. (1968). Biochim. Biophys. Acta, 167, 308.

Kuby, S.A. and Noltmann, E.A. (1962). In "The Enzymes", Vol. VI, p.515. Ed. by Boyer, P.D., Lardy, H. and Myrback, K. Academic Press : New York and London.

Kunitz, M. (1952). J. Gen. Physiol., 35, 423.

Kutscher, F. and Ackermann, D. (1926). Z. Biol., 84, 181, see Watts (1968).

Lacombe, G., Thiem, N.v. and Thoai, N.v. (1969). Europ. J. of Biochem., 9, 237.

Lineweaver, H. and Burk, D. (1934). J. Am. Chem. Soc., 56, 658.

Lumry, R. (1959). In "The Enzymes", Vol. I, p.157. Ed. by Boyer, P.D., Lardy, H. and Myrbäck, K. Academic Press : New York and London.

Lumry, R., Matsumiya, H., Bovey, F.A. and Kowalsky, A. (1961). J. Phys. Chem., 65, 837.

Luz, Z. and Meiboom, S. (1964). J. Chem. Phys., 40, 2686.

Mahowald, T.A., Noltmann, E.A. and Kuby, S.A. (1962). J. Biol. Chem., 237, 1535.

Marcus, F. and Morrison, J.F. (1964). Biochem. J., 92, 429.

Meister, A. (1965). In "Biochemistry of the Amino Acids", Vol. 1, 2nd ed. Academic Press : New York and London.

Melchior, J.B. (1965). Biochemistry, 4, 1518.

Michaelis, L. and Menten, M.L. (1913). Biochem. Z., 49, 333, see Segal (1959).

Mildvan, A.S. and Cohn, M. (1963). Biochemistry, 2, 910.

Mildvan, A.S. and Cohn, M. (1965). J. Biol. Chem., 240, 238.

Mildvan, A.S., Cohn, M. and Leigh, J.S. (1967). In "Magnetic Resonance in Biological Systems", p.113. Ed. by Ehrenberg, E., Malmström, B.G. and Vänngård, T. Pergamon Press, Oxford.

Moreland, B., Watts, D.C. and Virden, R. (1967). Nature, 214, 458.

Morrison, J.F. (1968). Analyt. Biochem., 24, 106.

Morrison, J.F. and Cleland, W.W. (1966). J. Biol. Chem., 241, 673.

Morrison, J.F. and Ennor, A.H. (1960). In "The Enzymes", Vol. II, p.89. Ed. by Boyer, P.D., Lardy, H. and Myrbäck, K. Academic Press : New York and London.

Morrison, J.F., Griffiths, D.E. and Ennor, A.H. (1957). Biochem. J., 65, 143.

Morrison, J.F. and James, E. (1965). Biochem. J., 97, 37.

Morrison, J.F. and O'Sullivan, W.J. (1965). Biochem. J., 94, 221.

Morrison, J.F., O'Sullivan, W.J. and Ogston, A.G. (1961). Biochim. Biophys. Acta, 52, 82.

Morrison, J.F. and Uhr, M.L. (1966). Biochim. Biophys. Acta, 122, 57.

Morrison, J.F. and White, A. (1967). Europ. J. of Biochem., 3, 145.

Mourad, N. and Parks, R.E., Jnr. (1965). Biochem. Biophys. Res. Commun., 19, 312.

Mourad, N. and Parks, R.E., Jnr. (1966). J. Biol. Chem., 241, 271.

Needham, D.M., Needham, J., Baldwin, E. and Yudkin, J. (1932). Proc. Roy. Soc. (London), B, 110, 260.

Noat, G., Ricard, J., Borel, M. and Got, C. (1968). Europ. J. of Biochem., 5, 55.

Noda, L. (1958). J. Biol. Chem., 232, 237.

Noltmann, E.A., Gubler, C.J. and Kuby, S.A. (1961). J. Biol. Chem., 236, 1225.

Norman, A.W., Wedding, R.T. and Black, M.K. (1965). Biochem. Biophys. Res. Commun., 20, 703.

O'Sullivan, W.J. and Cohn, M. (1966a). J. Biol. Chem., 241, 3104.

O'Sullivan, W.J. and Cohn, M. (1966b). J. Biol. Chem., 241, 3116.

O'Sullivan, W.J. and Cohn, M. (1968). J. Biol. Chem., 243, 2737.

O'Sullivan, W.J. and Noda, L. (1968). J. Biol. Chem., 243, 1424.

O'Sullivan, W.J. and Perrin, D.D. (1964). Biochemistry, 3, 18.

O'Sullivan, W.J., Virden, R. and Blethen, S.L. (1969). Europ. J.of Biochem., 8, 562.

Ottolenghi, P. (1964). Compt. Rend. Trav. Lab. Carlsberg, 34, 237.

Packman, P.M. and Jakoby, W.B. (1965). Biochem. Biophys. Res. Commun., 18, 710.

Packman, P.M. and Jakoby, W.B. (1967). J. Biol. Chem., 242, 2075.

Parry, M.J. and Walker, D.G. (1966). Biochem. J., 99, 266.

Parry, M.J. and Walker, D.G. (1967). Biochem. J., 105, 473.

Peanasky, R., Kuby, S.A. and Lardy, H. (1957). In "Biochemical Preparations", Vol. 5, p.9. Ed. by Shemin, D. John Wiley and Sons, New York.

Pelletier, S. (1964). In "Stability Constants of Metal-Ion Complexes" compiled by Sillen, I.G. and Martell, A.E. Special publication No. 17, The Chemical Society, London.

Perry, S.V. (1954). Biochem. J., 57, 427.

Perutz, M.F. (1969). Europ. J. of Biochem., 8, 455.

Plowman, K.M. and Cleland, W.W. (1967). J. Biol. Chem., 242, 4239.

Pradel, L.A. and Kassab, R. (1968). Biochim. Biophys. Acta, 167, 317.

Pradel, L.A., Kassab, R., Der Terrossian, E. and Thoai, N.v. (1965). C.R. Acad. Sci. Paris, t. 260, 3212.

Pradel, L.A., Kassab, R., Regnouf, F. and Thoai, N.v. (1964). Biochim. Biophys. Acta, 89, 255.

Reynard, A.M., Hass, L.F., Jacobsen, D.D. and Boyer, P.D. (1961). J. Biol. Chem., 236, 2277.

Rhoads, D.G. and Lowenstein, J.M. (1968). J. Biol. Chem., 243, 3963.

Roa, D.R. and Oesper, P. (1961). Biochem. J., 81, 405.

Robin, Y., Klotz, C. and Thoai, N.v. (1969). Biochim. Biophys. Acta, 171, 357.

Robin, Y. and Roche, J. (1954). C.R. Soc. Biol. Paris, 148, 1783.

Robin, Y. and Thoai, N.v. (1962). Biochim. Biophys. Acta, 63, 481.

Roche, J. and Robin, Y. (1954). C.R. Soc. Biol. Paris, 148, 1541.

Rosenberg, H., Ennor, A.H. and Morrison, J.F. (1956). Biochem. J., 63, 153.

Ruiz Cruz, J., Virden, R. and Watts, D.C. (1963). Biochem. J., 89, 110P.

Rydon, H.N. and Smith, P.W.G. (1952). Nature, 169, 922.

Ryle, A.P., Sanger, F., Smith, L.F. and Kitai, R. (1955). Biochem. J., 60, 541.

Sasa, T. and Noda, L. (1964). Biochim. Biophys. Acta, 81, 270.

Schoellman, G. and Shaw, E. (1963). Biochemistry, 2, 252.

Schwartz, J.H. (1963). Ph.D. Thesis, Rockefeller Institute, N.Y.

Segal, H.L. (1959). In "The Enzymes", Vol. I, p.1. Ed. by Boyer, P.D., Lardy, H. and Myrbäck, K. Academic Press : New York and London.

Smith, E. and Morrison, J.F. (1969). J. Biol. Chem., 244, 4224.

Smithies, O. (1955). Biochem. J., 61, 629.

Spackman, D.H., Stein, W.H. and Moore, S. (1958). Analyt. Chem., 30, 1190.

Su, S. and Russell, P.J., Jnr. (1968). J. Biol. Chem., 243, 3826.

Szorenyi, E.T., Dvornikova, P.D. and Degtyar, R.G. (1949). Dokl. Akad. Nauk., S.S.S.R., 67, 341.

Thoai, N.v., Der Terrossian, E., Pradel, L.A., Kassab, R., Robin, Y., Landon, M.F., Lacombe, G. and Thiem, N.v. (1968). Bull. Soc. Chim. Biol., 50, 63.

Thoai, N.v., Kassab, R. and Pradel, L.A. (1965). Biochim. Biophys. Acta, 110, 532.

Thoai, N.v. and Robin, Y. (1954). Biochim. Biophys. Acta, 14, 76.

Thoai, N.v., Roche, J., Robin, Y. and Thiem, N.v. (1953). Biochim. Biophys. Acta, 11, 593.

Thoai, N.v., Zappacosta, S., Robin, Y. and Roche, J. (1963). C.R. Soc. Biol. Paris, 157, 769.

Tietz, A. and Ochoa, S. (1959). Arch. Biochem. Biophys., 78, 477.

Toews, C.J. (1966). Biochem. J., 100, 739.

Uhr, M.L. (1966). Ph.D. Thesis, Australian National University.

Uhr, M.L., Marcus, F. and Morrison, J.F. (1966). J. Biol. Chem., 241, 5428.

Virden, R. and Watts, D.C. (1964). Comp. Biochem. Physiol., 13, 161.

Virden, R. and Watts, D.C. (1966a). Biochem. J., 99, 159.

Virden, R. and Watts, D.C. (1966b). Biochem. J., 99, 162.

Virden, R., Watts, D.C. and Baldwin, E. (1965). Biochem. J., 94, 536.

Virden, R., Watts, D.C., Watts, R.L., Gammack, D.B. and Raper, J.H. (1966). Biochem. J., 99, 155.

Walaas, E. (1958). Acta Chem. Scand., 12, 528.

Walter, C. and Frieden, E. (1963). In "Advances in Enzymology", Vol. XXV, p.167. Ed. by Nord, F.F. Interscience Publishers : New York and London.

Watts, D.C. (1968). In "Advances in Comparative Physiology and Biochemistry", Vol. 3, p.71. Ed. by Lowenstein, O. Academic Press : New York and London.

Watts, D.C., Rabin, B.R. and Crook, E.M. (1961). Biochim. Biophys. Acta, 48, 380.

Wood, T. (1963a). Biochem. J., 87, 453.

Wood, T. (1963b). Biochem. J., 89, 210.

Woolf, B. (1931). Biochem. J., 25, 342.

Wratten, C.C. and Cleland, W.W. (1963). Biochemistry, 2, 935.

Yagi, K. and Mase, R. (1962). J. Biol. Chem., 237, 397.

Yagi, K. and Noda, L. (1960). Biochim. Biophys. Acta, 43, 249.

Yudkin, W.H. (1954). J. Cell. Comp. Physiol., 44, 507.

Zewe, V. and Fromm, H.J. (1965). Biochemistry, 4, 782.

Zewe, V., Fromm, H.J. and Fabiano, R. (1964). J. Biol. Chem., 239, 1625.